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POLYMER MODIFICATION

Abstract:

A process for producing adducts of a polymer and a target material which process comprises the steps of (a) reacting either (i) an activating compound of formula (I) X-AM with a polymer of formula (II) (C)_c POL - Gg so as to form (ii) a sulphonate ester-activated polymer of formula (III) (C)_c POL - (AM)g; (b) reacting the sulphonate ester-activated polymer of formula (III) or (III') with the target material and (c) recovering the adduct of the polymer and the target material, in which process: (i) the polymer of formula (II) is dry as determined by benzene distillation, (ii) the reaction of the compound of formula (I) or (I') with the polymer of formula (II) is conducted in an organic solvent which is inert to the reagents and to the product of formula (III) or (III') and is anhydrous as obtainable using molecular sieves of 0.3nm; (iii) the reaction of the compound of formula (I) or (I') with the polymer of formula (II) is conducted in a reaction vessel from which water is excluded; (iv) the sulphonate ester-activated polymer of formula (III) or (III') so produced is recovered and either used directly in step (b) or stored, prior to use in step (b), in the presence of a desiccating agent more hygroscopic than the product of formula (III) or (III'); and (v) the reaction of the sulphonate ester-activated polymer with the target material is conducted in a non-denaturing medium and non-denaturing temperature with respect to the target material.

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(21) International Application Number: PCT/GB94/01844 (22) International Filing Date: 23 August 1994 (23.08.94) (30) Priority Data: 9317618.8 24 August 1993 (24.08.93) GB (71) Applicant (for all designated States except US): ROYAL FREE HOSPITAL SCHOOL OF MEDICINE [GB/GB]; University of London, Rowland Hill Street, London NW3 2PF (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): FRANCIS, Gillian, Elizabeth [GB/GB]; Molecular Cell Pathology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB). FISHER, Derek [GB/GB]; Molecular Cell Pathology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB). DELGADO, Cristina [GB/GB]; Molecular Cell Pathology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB). MALIK, Farooq [GB/GB]; Molecular Cell Pathology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB).	(74) Agents: CRESSWELL, Thomas, Anthony et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB). (81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: POLYMER MODIFICATION (57) Abstract A process for producing adducts of a polymer and a target material which process comprises the steps of (a) reacting either (i) an activating compound of formula (I) X-AM with a polymer of formula (II) (C) _n POL - G _g so as to form (ii) a sulphonate ester-activated polymer of formula (III) (C) _n POL - (AM) _g ; (b) reacting the sulphonate ester-activated polymer of formula (III) or (III') with the target material and (c) recovering the adduct of the polymer and the target material, in which process: (i) the polymer of formula (II) is dry as determined by benzene distillation, (ii) the reaction of the compound of formula (I) or (I') with the polymer of formula (II) is conducted in an organic solvent which is inert to the reagents and to the product of formula (III) or (III') and is anhydrous as obtainable using molecular sieves of 0.3nm; (iii) the reaction of the compound of formula (I) or (I') with the polymer of formula (II) is conducted in a reaction vessel from which water is excluded; (iv) the sulphonate ester-activated polymer of formula (III) or (III') so produced is recovered and either used directly in step (b) or stored, prior to use in step (b), in the presence of a desiccating agent more hygroscopic than the product of formula (III) or (III'); and (v) the reaction of the sulphonate ester-activated polymer with the target material is conducted in a non-denaturing medium and non-denaturing temperature with respect to the target material.		

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Polymer Modification

The present invention relates to a process for direct covalent bonding of polymer moieties to target molecules in order to improve or modify the biological properties of the target molecules. The invention also relates to certain products which may be produced by this process and to intermediates useful in the process as well as to methods of use of the products of the process.

Covalent attachment of polyethylene glycol(PEG) and other polymers to proteins is well known to convey many benefits which improve the pharmacological and some physiological properties of proteins (reviewed in Nucci et al (1991) Advanced Drug Delivery Reviews, 6, 133-151; Zalipsky & Lee (1992) Biomedical Applications of Polyethylene Glycol Chemistry (ed. by J.M. Harris), Plenum, New York; Fuertges & Abuchowski, Journal of Controlled Release, 11, 139-148 1990).

There are many methods for achieving covalent coupling of polymers like PEG to proteins. All consist of a method of activating the polymer by attachment of a group or groups herein called an "activating moiety" or by converting a terminal moiety of the polymer into an "activating moiety" and a second step where the polymer couples to the target molecule, usually via a residual portion of the "activating moiety" herein called the "coupling moiety".

Typical targets are proteins which contain one or more reactive groups on which the attachment of the coupling moiety or polymer itself can take place, such as primary and secondary amino groups, thiol groups and aromatic hydroxy groups. Alternatively, the protein molecule has been modified so that it contains suitable groups. Similar techniques are applied in coupling PEG and other polymers to other types of target molecule.

Examples of known techniques are:

- Cyanuric Chloride Methods: USP 4 179 337 Davis et al; USP 4 301 144 Iwashita & Ajisaka; USP 4 261 973 Lee & Sehon; EP-A-0 098 110 Hiratani; USP 4 495 285 Shimitzu; USP 4 609 546 Hiratani; WO 86/04145 Tomasi; EP-A-0 210 761
- 5 Miyata et al; WO 90/06952 Ishikawa et al; GB 2 238 959A Sehon et al; Abuchowski et al (1977a), Journal of Biological Chemistry, 252, 3578-3581, Abuchowski et al (1977b), Journal of Biological Chemistry, 252, 3582-3586.
- 10 Succinimidyl Active Ester Methods: Boccu et al (1983) Z. Naturforsch, 38c 94-99; Abuchowski et al (1984) Cancer Biochem. Biophys., 7, 175-186; Leonard et al (1984) Tetrahedron, 40, 1585-1590; USP 4 412 989 Iwashita et al; WO 86/04145 Tomasi; WO 87/00056 Katre & Knauf; EP-A-0 247
- 15 860 Katre et al; EP-A-0-260 098 Inada et al; WO 89/06546 Shadle et al; Katre et al (1987) Proc. Natl. Acad. Sci. USA, 84, 1487-1491.
- 20 Carbonyldiimidazole Method: Beauchamp et al (1983) Analytical Biochemistry, 131, 25-33; EP-A-0 154 432 Sawai et al.
- 25 Phenylchloroformate Methods: Veronese et al (1985) Appl. Biochem. Biotechnol., 11, 141-152; WO 89/06546 Shadle et al; WO 90/15628 Groves et al.
- 30 PEG-Succinate Mixed Anhydride Methods: Ahlstedt et al (1983) Int. Arch. Allergy Appl. Immunol., 71, 228-232; Richter and Akerblom (1983) Int. Arch. Allergy Appl. Immunol, 70, 124-131; Richter & Akerblom (1984) Int. Arch. Allergy Appl. Immunol, 74, 36-39; Lee and Sehon (1978) Immunol. Rev, 41, 200-247; USP 4 261 973 Lee and Sehon.
- 35 Organic Sulphonyl Halide Methods: USP 4 415 665 Mosbach & Nilsson; Delgado et al (1990) Biotechnology and

Applied Biochemistry, 12, 119-128; WO 90/04650 Francis et al; WO 90/04606 Delgado et al; WO 90/04384 Fisher.

- PEG-Aldehyde Methods: Harris et al (1989) Separations
5 Using Aqueous Phase Systems. Applications in Cell Biology and Biotechnology (ed. by D. Fisher and I.A. Sutherland), p.203. Plenum Press, London; Harris et al (1991) Water-Soluble Polymers (ed. by S.W. Shalaby, C.L. McCormick and G.B. Butler), American Chemical Society, Washington, D.C.
10 USP 4 002 531 Royer; EP-A-0-251 304 Minami et al; WO 90/05534 Capon et al.

- PEG-Maleimide and Related Methods: Goodson & Katre
15 (1990) Biotechnology, 8, 343-346.

Phenylglyoxal Method: EP-A-0 340 741 Maeda et al.

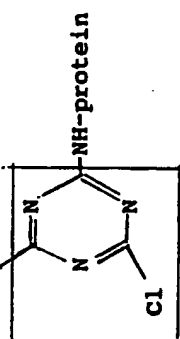
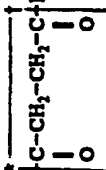
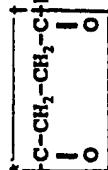
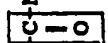
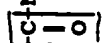
- Succinimide Carbonate Method: WO 90/13540 Zalipsky;
20 WO 91/07190 Nho et al;

Cyanogen Bromide Method: USP 4 301 144 Iwashita & Ajisaka.

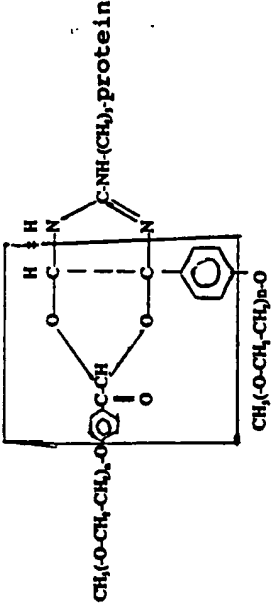
- Poly-PEG Maleic Acid Anhydride Method: Yoshimoto et
25 al (1987) Biochemical and Biophysical Research Communications, 148, 876-882.

The key features of the methods are summarised in Table 1.

Table 1

Method	Coupling Moiety (Boxed) and general structure of adduct	Bond	Co-Product	Recommended Coupling Reaction
Cyanuric Chloride	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}$  NH-protein		HCl	1h, RT, pH 9.2(3) 1h, RT, pH 9.8 (4) 1h, 37°C, pH10(5)
Succinimidyl active ester (succinimidyl succinate)	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}$  NH-protein	† amide bond * ester bond	N-hydroxy-succinimide	30 min, pH 7(6) 1h, RT, pH8.25(7) 30min, RT, pH9(8)
PEG-succinate mixed anhydride	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}$  NH-protein	† amide bond * ester bond	ester plus ester-modified protein	30 min, RT plus overnight 0°C pH 8.7 (9)
Phenylchloroformates	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}$  NH-protein	carbamate	substituted phenol	1-5h, pH8.3-9.3(10)
Carbonyldiimidazole	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}$  NH-protein	carbamate	imidazole	24-72h, 4°C, pH8.5 (11,12)

Method	Coupling Moiety (Boxed) and general structure of adduct	Bond	Co-Product	Recommended Coupling Reaction
Succinimide carbonate	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\text{C} \begin{array}{c} \text{ } \\ \text{O} \end{array} \text{NH-protein}$	carbamate	N-hydroxy-succinimide	30min, pH9.3 (13)
Poly(PEG-MA) anhydride	$\begin{array}{c} \text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\text{CH}_2-\text{CH} \\ \text{CH-COOH} \\ \text{CH-CO-NH-protein} \\ \text{CH}_2 \\ \text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\text{CH}_2-\text{CH} \\ \text{CH-COOH} \\ \text{CH-COOH} \end{array}$	amide bond		2h, 25°C, pH8.5 (14)
PEG-maleimide	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{NH}-\text{C} \begin{array}{c} \text{ } \\ \text{O} \end{array} \text{N} \begin{array}{c} \text{O} \\ \text{C} \end{array} \begin{array}{c} \text{O} \\ \text{C} \end{array} \text{S-protein}$	amide bond		several hours, RT, pH5-7 (15) 2hr, RT, pH5.5 (16)

Method	Coupling Moiety (Boxed) and general structure of adduct	Bond	Co-Product	Recommended Coupling Reaction
PEG-acetaldehyde	no coupling moiety present or ethylene oxide unit $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{protein}$	‡ secondary amine	oxidised cyano-borohydride	Not indicated (17)
Sulphonic halogenide	no coupling moiety present $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{NH}-\text{protein}$	‡ secondary amine	sulphonic acid	1h, RT, pH 7.5 (18)
Phenyl glyoxal		‡ secondary amine		pH 8.5, 7hr, RT (19)

Method	Coupling Moiety (Boxed) and general structure of adduct	Bond	Co-Product	Recommended Coupling Reaction
Cyanogen bromide	This process potentially provides three products $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\boxed{\text{C}(=\text{O})-\text{NH}-\text{protein}}$ and $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\boxed{\text{C}(=\text{O})-\text{NH}-\text{protein}}$ and $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\boxed{\text{C}(=\text{O})-\text{NH}-\text{protein}}$	carbamate	NH_3	16h, 0°C, pH 7.5 (20)
		imido carbonate	NH_3	
		-	None	
Amine acylation	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{O}-\boxed{\text{C}(=\text{O})-\text{NH}-\text{phospholipid}}$	† amide	PEG-carboxylate	3h, 65°C, benzene (21)

Method	Coupling Moiety (Boxed) and general structure of adduct	Bond	Co-Product	Recommended Coupling Reaction
PEG-propion-aldehyde	$\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n \boxed{\text{S}-(\text{CH}_2)_2}$ or $\text{CH}_3(-\text{O}-\text{CH}_2-\text{CH}_2)_n \boxed{\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2}$ $\uparrow \quad \uparrow$ $\text{NH-protein} \quad \text{NH-protein}$	† secondary amine	oxidised cyano-borohydride	1H, RT, pH9 (22)

Notes

1. cf. 2,4,5-trichlorophenol from MPEG 2,4,5-trichlorophenylcarbonate; p-nitrophenol from MPEG-p-nitrophenylcarbonate.
2. Room temperature
3. Abuchowski (1977a)
4. Pyatak *et al.* (1980) Res, Commun. Chem. Pathol. Pharmacol., 29, 113-127.
5. Yoshimoto, *et al.* (1986) Jpn. J. Can. Res., 77, 1264-1270.
6. Abuchowski (1984).

- 9 -

Notes to Table 1 cont'd

7. Leonard (1984).
8. Katre (1987).
- 5 9. Wie et al. (1981) Int. Arch. Allergy Applied Immunol., 64,84
10. Veronese (1985).
11. Beauchamp (1983) and EP-A-O 154 432
12. Pizzo (1991) Advanced Drug Delivery Reviews, 6, 153-166.
13. Zalipsky (1992).
- 10 14. Yoshimoto (1987).
15. Aldwin and Nitecki, (1987) Analytical Biochemistry, 164, 494-501.
16. Goodson and Katre (1990).
17. Harris (1989).
- 15 18. Delgado (1990).
19. Maeda et al. (1989) EP-A-0340741
20. Iwashita et al. (1981) US-A-4301144
21. Sears (1983) EP-A-0072111
- 22 Harris et al. New polyethylene glycols for biomedical
- 20 applications, in Shalaby SW, McCormick CL, Butler GB (eds):
Water-soluble Polymers, Washington D.C., American Chemical
Society, 1991.

Methods previously disclosed suffer from one or more
25 of the following defects:

1. Substantial loss of biological activity (e.g.

- 10 -

20-95% loss of bio-activity) is frequently seen with the cyanuric chloride and carbonyldiimidazole methods and occasionally with phenylchloroformate and succinimidyl active ester methods.

5

2. The coupling of PEG (or other polymers) to proteins (or other target molecules) is, with few exceptions, in a manner which leaves part of the activating moiety, the coupling moiety, between the polymer and the target molecule. Of the above methods, only the organic sulphonyl halide methods and a PEG-acetaldehyde method (one of the PEG aldehyde methods [Harris (1989), Harris (1991)]) couple PEG directly without coupling moieties (see Table 1). With the exception of some PEG acetaldehyde methods where the coupling moiety is ethylene oxide (and thus indistinguishable from PEG itself) and the direct coupling methods above, all other coupling methods incorporate a coupling moiety distinct from the polymer and the target and are regarded herein as "indirect" coupling methods.

20 The incorporation of a coupling moiety generates further problems depending on the nature of the coupling moiety, thus

(i) some coupling moieties provide reactive groups capable of linking further molecules to the polymer-target construct via the coupling moiety (e.g. the triazine ring of the cyanuric chloride method, Leonard, M. et al., Tetrahedron, 40: 1585 (1984));

(ii) some coupling moieties provide an immunogenic/antigenic group (e.g. the triazine ring of the cyanuric chloride method);

(iii) some coupling moieties are potentially

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5 toxic or are themselves of unknown toxicity but derived from a compound known to be toxic (e.g. the triazine ring of the cyanuric chloride method and reagents in the phenylchloroformate method); and

(iv) some coupling moieties provide targets for enzymatic cleavage (see below).

10 3. Coupling in some instances is via an unstable bond liable to be cleaved by enzymes available in serum, plasma, cells or other biological materials or by procedures applied to the polymer-target product. This has two possible deleterious consequences,

15 (i) the polymer-target construct is degraded enzymatically or by the conditions required for subsequent reaction steps; the former occurs with methods generating amide and/or ester bonds (see Table 1); and

20 (ii) removal of the polymer alters the target molecule; this occurs with some succinimidyl active ester and mixed anhydride methods,

25 and either or both of these can occur.

30 4. Many methods recommend long coupling times and/or unphysiological pH, thus rendering some target molecules less active or inactive (cf. the carbonyl-diimidazole, cyanuric chloride, phenylchloroformate and some succinimidyl active ester methods (see Table 1)).

35 5. Many methods use activated polymer species and/or produce co-products which are toxic in a wide range

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of bioassays and which are potentially toxic in vivo if not separated from the product (e.g phenylchloroformate, cyanuric chloride methods).

5 6. Many methods produce products highly contaminated with either activated or inactivated polymer, causing problems in analytical methods like FPLC and toxicity in some bioassay systems (cf. the organic sulphonyl halide [Delgado (1990)] and phenylchloroformate
10 [Veronese (1985)] methods).

 7. Some methods are unsuitable for use in aqueous solution, thus limiting the target molecules to those which will tolerate non-aqueous conditions (cf. the organic
15 sulphonyl halide method using trifluoromethanesulphonyl chloride).

 8. Some of the activated polymer constructs are unstable, for instance being subject to hydrolysis during
20 either the activation or coupling reactions (cf. the phenylchloroformate method [Veronese (1985)]). The PEG-acetaldehyde is sensitive to decomposition under basic conditions and can give irreproducible results [Harris et al. (1991)].

25

The present inventors have previously described PEGylation by an organic sulphonyl halide method using 2,2,2-trifluoroethanesulphonylmonomethoxy PEG (TMPEG) as follows:

30

 1. WO 90/04606 discloses a method for fractionation of PEG-proteins formed by the TMPEG method (discussed below) and an individual adduct, PEG-gm-CSF. The fractionation method is unrelated to the present invention.
35 The PEG-protein products of the processes disclosed in this application, and specifically disclosed materials such as

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the PEG-gm-CSF adduct, are excluded from the present invention.

2. PCT WO 90/04650 discloses a method to separate DNA/protein complexes using PEG adducts formed by the TMPEG in which PEG acts as an affinity ligand. These complexes are also excluded from the present invention.

3. PCT WO 90/04384 discloses the TMPEG method for attaching PEG to liposomes. Such PEGylated liposomes are excluded from the present invention. It should be noted that the objective of coupling PEG to liposomes in this patent was to improve half life and liposome stability.

The TMPEG method mentioned above, for instance in WO-A-90/04606, comprises activation of monomethoxy PEG (MPEG) with 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) to produce tresyl MPEG (TMPEG) which is subsequently reacted with the target protein molecule to produce monomethoxy products. The same technique is described in WO 90/04650 for coupling monomethoxy PEG moieties to DNA/protein complexes and in WO/04384 for coupling monomethoxy PEG moieties to liposomes. Further investigation of the TMPEG method has revealed that the reactivity of the activated polymer, TMPEG, is severely impaired necessitating the use of uneconomically large ratios of TMPEG to the target molecules and has exposed the rather poor yield of the final products and the low purity thereof, necessitating extensive purification and thereby further reducing the overall yield.

The present inventors have addressed these difficulties in further research and have identified a number of factors which, in combination, contribute to the poor performance of the TMPEG process. This has allowed development of an improved technique for producing PEG adducts. The technique has been extended to include additional technical developments permitting more facile

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formation of complex adducts of PEG and other polymers and the formulation of adducts of a wider range of target molecules.

The present invention relates to an improved process
5 for producing polymer:target molecule adducts which are directly covalently linked without any intervening coupling moiety residue from the activating group on the polymer and in which the link between polymer and target moieties is non-immunogenic, non-antigenic, non-toxic and non-
10 biodegradable, this objective being achieved by careful control of the properties of certain of the reagents and the reaction conditions. The coupling step may be performed in aqueous media and occurs rapidly thus minimising damage to fragile target species.

15

- 15 -

Accordingly the invention provides a process for producing adducts of a polymer and a target material which process comprises the steps of

5 (a) reacting either

(i) an activating compound of formula (I)



10

wherein

-AM is an activating sulphonyl ester moiety optionally bearing a group for covalent bonding to a solid support, the solvolysis substituent constant of the group -AM being less than that of the trifluoromethane sulphonate group, and

15

X is a leaving group

20 or

(ii) reacting a solid support bearing moieties of formula (I')



25

wherein

-AM'- is activating sulphonyl ester moiety covalently bound to the solid support, the group -AM'- being such that the solvolysis substituent constant of the group -AM'- is less than that of the trifluoromethane sulphonate group, and

30

X is as defined above

35

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with a polymer of formula (II)



5 wherein

POL is a polymer moiety of valency $c+g$,

C is a capping group and c is zero or a
positive number and

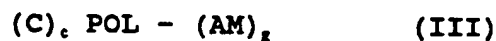
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G is a terminal hydroxyl group reactive with
the compound of formula (I) and g is a positive
number

15 so as to form

(i) a sulphonate ester-activated polymer of
formula (III)

20



wherein

C, POL, -AM, c and g are as defined above and
the group(s) -AM are linked to termini of the
25 polymer as sulphonate esters of terminal
hydroxyl groups

or (ii) a solid support-bound, sulphonate ester-activated
polymer of formula (III')

30



35

- 17 -

wherein

C, POL, -AM'-, c and g are as defined above,

5 SS is a solid support and z is the number of
sulphonate ester-activated polymer moieties on
the solid support

10 and the groups -AM'- are linked to the termini
of the polymer as sulphonate esters of terminal
hydroxyl groups,

and, when -AM bears a group for covalent bonding to a solid
support, reacting the sulphonate ester-activated polymer of
formula (III) with a solid support to form a solid support-
15 bound, sulphonate ester-activated polymer of formula (III')
as defined above

20 (b) reacting the sulphonate ester-activated polymer of
formula (III) or (III') with the target material and

(c) recovering the adduct of the polymer and the target
material,

in which process:

25

(i) the polymer of formula (II) is dry as
determined by benzene distillation,

30 (ii) the reaction of the compound of formula
(I) or (I') with the polymer of formula
(II) is conducted in an organic solvent
which is inert to the reagents and to the
product of formula (III) or (III') and is
anhydrous as obtainable using molecular
35 sieves of 0.3nm;

(iii) the reaction of the compound of formula

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- (I) or (I') with the polymer of formula (II) is conducted in a reaction vessel from which water is excluded;
- (iv) the sulphonate ester activated polymer of formula (III) or (III') so produced is recovered and either used directly in step (b) or stored, prior to use in step (b), in the presence of a desiccating agent more hygroscopic than the product of formula (III) or (III'); and
- (v) the reaction of the sulphonate ester-activated polymer with the target material is conducted in a non-denaturing medium and non-denaturing temperature with respect to the target material.

The compounds of formula (I) and solid supports bearing moieties of formula (I'), the polymer moiety POL in the polymers of formulae (II), (III) and (III'), the sulphonate ester-activating moieties -AM and -AM'-, the reactive groups X and G and the capping groups C will be described in more detail below. First, some variants of the overall process will be described:

In general the process of the invention is conducted in the liquid phase, using a compound of formula (I) in step (a), in solvents and reaction media as defined above. However it is also possible to conduct the reaction at a solid:liquid interface by using a solid support-bound, sulphonate ester-activated polymer of formula (III'). In this latter case the reaction can be conducted on, for instance, a column of solid support-bound sulphonate ester-activated polymer with other reagents and reaction media being passed through the column.

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The objective of performing the reaction on a solid support is that although the target is exposed to a molar excess of sulphonate ester-activated polymer, only those polymer moieties which become attached to the target are eluted from the solid support and the potentially toxic co-product remains attached to the column. The flow rate and column length provide a means of controlling the degree of modification. Column media can also be selected to achieve differential elution of targets modified to different extents.

Binding of the sulphonate ester-activated polymer to the solid support is achieved via the activating ester-sulphonyl moiety -AM in one of two ways:

1. The process is conducted using a compound of formula (I) which is an activating sulphonyl compound having a binding substituent for later linking to the solid support. In this variant, the reaction between the compound of formula (I) and the polymer of formula (II) is conducted in the solution phase and is followed by the step of binding the product of formula (III) to the solid support. The reaction in step (b) between the solid support-bound, sulphonate ester-activated polymer of formula (III') and the target material is then performed at the solid:liquid interface.

2. The process is conducted using a solid support bearing activating sulphonyl moieties of formula (I') in step (a). In this case the reaction of the compound of formula (I') with the polymer material of formula (II) is conducted at the solid:liquid interface and directly forms the solid support-bound, sulphonate ester-activated polymer of formula (III'). The solid support bearing moieties of formula (I') is produced by reacting a compound of formula (I) or a precursor therefor which bears a group for covalent bonding to a solid support with the solid support

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as a preliminary to step (a) of the present process. Step (b) is again conducted at the solid:liquid interface as in (1) above.

5 The benefit of conducting the reaction(s) at a solid:liquid interface is primarily seen in the greater purity of the products since unreacted sulphonate ester-activated polymer is left on the solid support as are the reaction products other than the polymer:target adduct.

10 The present invention therefore provides the following particular embodiments of the process:

1. A process as described above comprising the
15 steps of reacting a compound of formula (I) with a polymer of formula (II) in the liquid phase to produce a sulphonate ester-activated polymer of formula (III) and reacting the sulphonate ester-activated polymer with the target material in the liquid phase.

20 2. A process as described above comprising the steps of reacting a compound of formula (I) wherein -AM is an activating sulphonyl moiety having one or more groups for covalent bonding to a solid support with a polymer of
25 formula (II) in the liquid phase, coupling the thus-produced sulphonate ester-activated polymer to a solid support material via the binding groups of the moieties -AM and reacting the target material with the thus-produced solid support-bound, sulphonate ester-activated polymer. In
30 this embodiment step (c) is achieved when the adduct is released from the solid support, eluted and thus recovered.

3. A process as described above comprising the steps of reacting a solid support bearing moiety of formula
35 (I') with a polymer of formula (II) so as to form a solid support-bound, sulphonate ester-activated polymer of

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formula (III') and reacting the solid support-bound, sulphonate ester-activated polymer with the target material.

5 In further variants of the process of the invention, which can be conducted in conjunction with any of the foregoing variants, the proportion of the groups -AM on the sulphonate ester-activated polymer which react with the (initial) target material is controlled such that in at
10 least a proportion of the molecules of polymer:target adduct produced there remain unreacted activating sulphonyl moieties -AM. Such unreacted reactive groups -AM may then be reacted with one or more different further target materials so as to form materials polymer:[target], wherein
15 n is 2 or more and the initial and further target entities are different; or they are reacted with more of the initial target material so as to form materials polymer:[target], wherein the target entities are all the same; or they are reacted with further unreacted groups in the initial target
20 entity of the polymer:target adduct (for instance under conditions which hinder intermolecular reactions, such as high dilution) such that the polymer moieties are bonded at two or more positions to the target entity.

 It will be appreciated that, since the polymer and/or
25 the target materials may be multivalent, it is possible by the process of the invention to produce a variety of polymer:target structures. By way of example, a univalent polymer and univalent target produce 1:1 adducts; a bivalent target and a univalent polymer may form adducts
30 wherein the target entities bear two polymer moieties whereas a bivalent polymer and a univalent target may produce species where two target entities are linked to a single polymer moiety; use of higher-valent polymers can lead to the formation of clusters of target entities bound
35 to a single polymer moiety whereas higher-valent targets may become encrusted with a plurality of polymer moieties.

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In general the target moieties are likely to have more than one reactive group which will react with the activated polymer and the possibility of forming complex structures must always be considered; when it is desired to form
5 simple structures such as 1:1 adducts of polymer and target, or to use bivalent polymers to form target:polymer:target adducts, it will be necessary to use predetermined ratios of activated polymer and target material, predetermined concentrations thereof and to
10 conduct the reaction under predetermined conditions (such as duration, temperature, pH etc.) so as to form a proportion of the described product and then to separate the described product from the other reaction products.

The necessary reaction conditions, proportions and
15 concentrations of the reagents can be obtained by relatively simple trial-and-error experiments with appropriate scaling-up as necessary. It will be appreciated that molar proportions and the concentrations of individual reagents may need to be adjusted on scaling up compared
20 with the optimal molar proportions and concentrations identified at the laboratory bench scale for production of a particular product; this may also require relatively simple trial-and-error experimentation. Purification and separation of the products is similarly achieved by
25 conventional techniques well known to those skilled in the art.

The present invention therefore provides the following particular embodiments of the process:

30

1. A process as described above comprising, in step (b), reacting, in predetermined molar ratio and at predetermined concentrations, sulphonate ester-activated polymer having two moieties -AM with a first target
35 material, so as to produce a sulphonate ester-activated 1:1 adduct of polymer and target material, then reacting the

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1:1 adduct with a second target material so as to produce a target¹:polymer:target² adduct, wherein the first and second target materials may be the same or different, and then recovering the adduct in accordance with step (c).

5

2. A process as described above comprising, in step (b), reacting, in predetermined molar ratio and at predetermined concentrations, sulphonate ester-activated polymer having only one moiety -AM or -AM'- with a target
10 material having more than one reactive group so as to form an adduct of the polymer and target having a preselected number of polymer molecules per molecule of target or, especially for macromolecular targets having a large number of reactive groups, so as to form an adduct in which a
15 preselected proportion of the reactive groups have been reacted with and linked to polymer molecules, and then recovering the adduct in accordance with step (c). The preselected number may be, for instance from 1 to 10, preferably from 1 to 5, for example 1, 2, 3 or 4. The
20 preselected proportion may be, for instance from 1 to 100 %, preferably from 5 to 95 %, for example, 10, 20, 30, 40, 50, 60, 70, 80, or 90%.

3. A process as described above comprising, in
25 step (b), reacting, in predetermined molar ratio and at predetermined concentrations, sulphonate ester-activated polymer having two or more activating sulphonyl moieties -AM with a target material having two or more reactive
30 adduct of polymer and target then changing the reaction conditions to hinder intermolecular reactions whilst permitting intramolecular reactions between groups -AM on the polymer and reactive groups on the target so as to form adducts of polymer and target having two or more covalent
35 bonds between the polymer and target moieties, and then recovering the adduct in accordance with step (c).

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These process variants may each be used in conjunction with any of the process variants described earlier relating to liquid phase and solid:liquid phase interface reactions.

- 5 The reagents used in the process of the invention will now be described starting with the compounds of formula (I):

ACTIVATING COMPOUNDS OF FORMULA (I) and SOLID SUPPORTS

10 BEARING MOIETIES OF FORMULA (I')

- 15 The leaving group X is intended to react with groups G on the polymer of formula (II) and, in the broadest sense, may be selected from any of the known leaving groups which will undergo such a reaction. However it is generally preferred that X is a halogen and most preferably X is chlorine.

- 20 Activating moieties, -AM and -AM'- are sulphonyl-type activating groups, and optionally have a binding substituent for subsequent linking to a solid support or are attached to a solid support. The groups -AM will be described below in general, followed by further information on binding substituents and linkage to solid supports.

The activating moieties -AM are moieties having the following properties:

1. They are sulphonyl groups; the derived
30 sulphonate esters are a large class of compounds with a broad range of solvolytic activities.

2. They have appropriate electron withdrawing groups on the sulphur. The suitability of such groups can
35 be estimated by linear free energy relationship plots. It has been observed [Crossland et al (1971) J.Amer.Chem.Soc.,

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93, 4217-4219] that substituent constants may be applied to the reactivity of groups such as 2,2,2-trifluoroethane-sulphonate (tresylate), toluenesulphonate (tosylate), and methanesulphonate (mesylate) groups; there is a correlation
5 observed in a Hammett plot between the rates of solvolysis of sulphonate ester and the nature of the substituent on the sulphur and although this correlation is only approximate, it allows the predication of gross effects of various sulphonyl substituents on solvolytic reaction
10 rates. This technique provides a distinction between those sulphonate ester groups which are too highly reactive to be of use in the process of the present invention (i.e. those having a substituent constant or solvolysis rate equal to or greater than that of the trifluoromethane sulphonate
15 (triflate) group) and those which are of use and provides a useful means of preliminary selection within the latter. Substituent constants must be less than the values for the trifluoromethyl group in trifluoromethanesulphonates, preferably they are equal to or greater than the values of
20 the tosyl group in tosylates [Crossland et al (1971)]. This can for example be achieved by the inclusion of fluorine atoms in alkyl substituents as in 2,2,2-trifluoroethane-sulphonate (tresyl) esters. By selecting -AM from compounds having this range of substituent constants, one is
25 selecting reagents with a predetermined range of reactivities. This gives a means of predicting: a) the rate of the coupling reaction and b) the tendency of -AM to undergo hydrolysis in aqueous solution. It is important that the two rates are below that of triflate and
30 preferably equal to or above that of tosylate since, if the reactivity is too low, the coupling time will be long with potential damage to the target and if it is too high, hydrolysis will compete significantly with the coupling reaction and may preclude its occurring adequately in
35 aqueous solution.

If the target molecule is one that can withstand

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organic solvents these may be used for the coupling reaction. However, where the target molecule can withstand a slow coupling reaction then compounds with lower Hammett substituent constants than the range given above can be
5 used.

3. They contain no groupings more susceptible to nucleophilic attack than the terminal carbon atom of the polymer moiety for instance PEG, or equivalent atom of any
10 other polymer moiety (i.e. the carbon or equivalent atom bonded to the oxygen of the sulphonate group of the group -AM). The consequence of this is that no "coupling moiety" (i.e. residual part of the activating moiety) lies between the polymer and the target molecules.

15

4. The compounds from which the groups -AM are derived should preferably be stable.

5. To minimise toxic effects, the groups -AM and
20 the compounds from which they are derived should preferably be of low toxicity in vitro and in vivo.

6. The compounds from which the groups -AM are derived should be readily separated from the product of
25 formula (III).

Preferred groups -AM are:

2,2,2-trifluoroethanesulphonyl,
pentafluorobenzenesulphonyl,
30 fluorosulphonyl,
2,4,5-trifluorobenzenesulphonyl,
2,4-difluorobenzenesulphonyl,
2-chloro-4-fluorobenzenesulphonyl,
3-chloro-4-fluorobenzenesulphonyl,
35 4-amino-3-chlorobenzenesulphonyl,
4-amino-3-fluorobenzenesulphonyl,

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o-trifluoromethylbenzenesulphonyl,
m-trifluoromethylbenzenesulphonyl,
p-trifluoromethylbenzenesulphonyl,
2-trifluoromethoxybenzenesulphonyl,
5 4-trifluoromethoxybenzenesulphonyl, and
5-fluoro-2-methylbenzenesulphonyl groups.

The most preferred group -AM is 2,2,2
trifluoroethane-sulphonyl.

It should be appreciated that the definition of the
10 activated polymers used in the present invention excludes
certain sulphonic acid esters of polymers which have
previously been used in "active ester" methods of
PEGylation such as the ester between the carboxylic acid of
the polymer and the hydroxyl of 4-hydroxy-3-nitrobenzene
15 sulphonic acid described in Katre et al. (1987).

The binding substituents for linking the group -AM to
a solid support are suitably groups which can be coupled to
conventional solid support materials, optionally via a
20 conventional spacer moiety, by well known techniques. A
preferred binding substituent is an aminophenyl group which
can be coupled to a solid support via the amino
substituent. Preferred compounds of formula (I) for
binding to solid supports are therefore sulphonyl halides
25 having an aminophenyl group bound to the sulphur atom,
possibly via other moieties, such as 2',3',5',6'-tetra-
fluoro-4-amino-phenyl-azobenzene-4'-sulphonic halides,
especially the chloride.

30 Solid support materials which may be used in the
process of the invention are entirely conventional and well
known to those skilled in the art. It is preferred that the
solid support is selected from silica, silconised glass,
agarose, Sepharose, polystyrene divinyl benzene copolymer
35 and polyacrylamide.

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Compounds of formula (I) and solid supports bearing groups of formula (I') may be produced by conventional techniques well known to those skilled in the art. Many of these compounds are commercially available.

5 Compounds of formula (I) which are intended for linking to solid supports are also readily available or are produced by conventional techniques; they may be coupled to solid supports by conventional techniques optionally with introduction of spacer groups. Alternatively, in view of
10 the reactivity of sulphonyl halides, it may be preferred to couple a precursor, such as a sulphonic acid, of the compound of formula (I) with the solid support or reagents for introducing a spacer group then to convert the bound precursor to the sulphonyl halide group.

15 An example of a compound of formula (I) bearing a group for covalent bonding to a solid support is tetrafluoro-4-amino-azobenzene-4'-sulphonyl chloride which can be generated in a three step procedure as described by Scouten et al (1991) Journal of Chromatography, 376, 289-
20 298 as follows:-

1. Tetrafluorosulphanilic acid can be prepared from tetrafluoroaniline.

25 2. Tetrafluoro-4-amino-azobenzene-4'-sulphonic acid is prepared from tetrafluorosulfanilic acid by diazotisation and reaction with aniline.

3. The sulphonic acid is converted to the
30 sulphonyl chloride by drying with thionyl chloride and then grinding with phosphorus pentachloride and phosphorus oxychloride.

Coupling of compounds of formula (I) bearing moieties
35 -AM having solid support binding groups or precursors thereof are coupled to a solid support, for instance by

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reacting the compound of formula (I) with the solid support, with a spacer group on the solid support, or with reagents to introduce a spacer group and coupling the spacer group to the solid support. There are many possible compounds suitable for use in forming spacer groups. The spacer needs to be inert and not damaging to the target material in the context of the complete solid-supported reagent since, for instance, polymer modification may alter the properties of the solid support. The spacer needs to conserve the electron withdrawing groups giving -AM'- an appropriate substituent constant as defined for soluble -AM polymer constructs. It is particularly important to avoid groups susceptible to nucleophilic attack. The selection of the spacer will also depend on the support selected and the properties defined above. The group on the sulphonyl activating moiety -AM for covalent bonding to the solid support will be capable of reacting with a reactive group on the solid support or spacer and will be chosen depending on the nature of the chosen solid support and any spacer group.

For instance, solid supports bearing aldehyde groups are reacted with an amino group on the compound of formula (I) in the presence of a non-nucleophilic base such as lithium isopropylamide so as to form a Schiff's base, followed by reduction of the double bond, for instance using an appropriate borohydride.

Suitable precursors of the compounds of formula (I) are readily available or can be produced by conventional techniques. Suitable solid support materials are also readily available; those which do not have groups capable of linking the precursor of the compound of formula (I) may readily be modified by conventional techniques in order to introduce the necessary functionalities, for instance siliconised glass (prepared as described by Mohr and Pommerening (1985) Affinity Chromatography, Practical and Theoretical Aspects, Marcel Dekker, Inc, New York and

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Basel) is activated using glutaraldehyde [Weston & Avrameas (1991) Biochemical and Biophysical Research Communications, 45, 1574-1580].

5 The compounds of formula (I) and solid supports bearing groups of formula (I') are readily hydrolysed and extremely labile and are preferably used when freshly prepared or, after preparation, are stored in air-tight containers such as glass ampoules prior to use.

10

POLYMERS OF FORMULA (II)

The polymers of formula (II) used in the present invention are all based on known and readily available polymers which generally already contain at least one group
15 G, reactive with the group X of the compound of formula (I). As polymers are almost inevitably mixtures, the value of g will be an average for the material as a whole but is preferably nearly or, more preferably, exactly integral, preferably with a high degree of homogeneity. For polymers
20 which have two or more reactive groups G it may be necessary or desirable to prevent reaction at a proportion of such groups by blocking them with a capping group C. The number of capping groups will also be an average for the material as a whole and is preferably nearly or, more
25 preferably, exactly integral, or is zero when no capping groups are required. The polymers, reactive groups G and capping groups C will be further described below.

The polymers that can be used are selected from the following which are all readily available to those skilled
30 in the art:

1. Homo- and heteropolymers, i.e. polymeric substances with repeating identical or non-identical subunits (homo-polymers and heteropolymers respectively)
35 such as:

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(i) Polyalkylene compounds; for example

a), polyalkylene oxides and glycols and their derivatives such as poly(oxymethylene), polyethyleneglycols and oxides and methoxypolyethyleneglycols and related homopolymers, such as polymethylethyleneglycol, polyhydroxypropyleneglycol, polypropyleneglycols and oxides, polymethylpropyleneglycol, and polyhydroxypropyleneoxide, which may be straight-chain or branched polymers (for example straight-chain polypropyleneglycols and branched-chain polypropyleneglycols) and derivatives of the above including ethers, for instance of polyethyleneglycol or polypropyleneglycol such as the monomethyl ethers, monocetyl ethers, mono-n-butyl ethers, mono-t-butylethers and monooleyl ethers, esters of polyalkyleneglycols with carboxylic acids such as the monobutyl esters and monostearyl esters, and dehydration condensation products of the polyalkyleneglycols with amines such as propylamine and stearyl amine;

b) polyvinyl compounds such as poly(vinylpyrrolidone), polyvinyl alcohol, poly(vinyl acetate) and the copolymer poly(vinyl acetate-co-vinyl alcohol), poly(vinylloxazolidone), poly(vinylmethyloxazolidone) and poly(vinyl methyl ether);

c) polyacrylic compounds such as poly(acrylic acid)s, poly(methacrylic acid)s, polyhydroxyethylmethacrylates, their amides such as poly(acrylamide) and poly(methacrylamide) and N-substituted derivatives of the amides such as poly(N,N-dimethylacrylamide), poly(N-isopropylacrylamide), poly(N-acetamidoacrylamide) and poly(N-acetamidomethacrylamide);

d) polyionic compounds such as poly(ethyleneimine), poly(ethylsulphonic acid), poly(silicic acid), poly(styrenesulphonic acid), poly(vinyl amine), poly(2-vinylpyridine) and its N-alkyl derivatives, poly(4-vinylpyridine) and its N-alkyl derivatives, poly(vinylsulphuric acid), poly(vinyl alcohol-co-vinyl

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- sulphuric acid), poly(diallyldimethylammonium chloride), poly[(dimethylimino) trimethylene(dimethylimino)hexamethylene dibromide)], poly(ethylenephosphonic acid), poly(maleic acid), poly(2-methacryloyloxyethane-1-sulfonic acid), poly(3-methacryloyloxypropane-1-sulfonic acid), poly(4-vinylbenzoic acid); poly(4-vinylbenzyl-trimethylammonium), poly[3-(vinylloxy)propane-1-sulphonic acid)], poly(4-vinylphenol), (poly[p-hydroxystyrene]), poly(4-vinylphenyl sulphuric acid), poly(2-vinyl-piperidine), poly(4-vinylpiperidine); poly(N-vinylsuccinamic acid) and ionizable synthetic copolymers;
- e) polyalkylene polyols such as polyoxyethylated glycerol, polyoxyethylated sorbitol (e.g polysorbates and polyoxyethylated glucose; and
- f) non-ionic surfactants such as polyoxyethylenealkylphenols (including Tritons), polyoxyethylenemercaptans, polyoxyethylenealkylamines and polyoxyethylenealkylamides;
- (ii) Polyamino acids or synthetic polypeptides such as, for example, polymers of D-glutamic acid and D-lysine, polylysine, polyalanine, polyglutamic acid, polyaspartic acid and polyproline;
- (iii) Polysaccharides, crosslinked products thereof and polysaccharide containing material including branched or unbranched polysaccharides, comprising saccharide monomers such as glucose, mannose, galactose, fucose, fructose, xylose, arabinose, glucuronic acid, sialic acid (neuraminic acid), galacturonic acid, mannuronic acid, D-glucosamine and galactosamine, which may be homopolysaccharides or

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- heteropolysaccharides, such as
- a) dextran and dextran derivatives including dextran sulphate, p-aminoethyl cross-linked dextran, and carboxymethyl dextran;
 - 5 b) cellulose and cellulose derivatives including methyl cellulose and carboxymethyl cellulose;
 - c) starches (amylopectin and amylose) and dextrans derived from starch, hydroxyethyl starches, agarose, ficoll or its carboxy methyl derivatives;
 - 10 d) glycosaminoglycan chains of proteoglycans such as hyaluronic acid, chondroitin sulphates, dermatan sulphate, heparin, heparin fragments, heparin oligosaccharides, heparan sulphate and keratan sulphate;
 - e) carbohydrate-containing side chains of
 - 15 glycoproteins and glycolipids such as gangliosides, globosides and sulphatides;
 - f) polymers of sugar alcohols such as polysorbitol and polymannitol; and
 - 20 g) other polysaccharides such as glycogen, glucans (e.g. laminaran), glycosaminoglycans, polysaccharide sidechains of glycoproteins or glycolipids, algal polysaccharides (e.g. alginic acid or polymannuronic acid and sulphated polysaccharides such as carrageenan and
 - 25 agar), pectins, plant gums, seed mucilages such as guaran, bacterial and fungal polysaccharides (e.g. xanthans, gellan, alginate, scleroglucan, schizophyllan, curdlan and pullulan);
 - 30 (iv) other organic polymers including polymers and copolymers of, for instance, amines, olefins, esters, acetal, polyamides, carbonates, ethers, phenylene sulphides, silicones, urea formaldehyde condensation
 - 35 products, phenol formaldehyde condensation products, urethanes,

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melamine formaldehydes, epoxy resins, acrylic resins, allyl resins, polyacrylic acid and carbomers; and

- 5 (v) inorganic polymers especially those inorganic polymers containing organic moieties, for example, silicates.

2. Block copolymers, i.e. copolymers formed by
10 combining appropriate blocks of the above polymers and include for instance block co-polymers of small alkoxy monomers, e.g. polyethylene/polypropyleneglycol (over 30 polyoxyethylene-polyoxypropylene copolymers are available on the market); block copolymers of ethylene and
15 maleic anhydride; block copolymers of polyalkylene glycols and polyvinylpyrrolidone or polyvinyl alcohol; block copolymers of polyoxyethylene and polyoxy-propylene (Pluronic); block copolymers of the ethers, esters or dehydration condensation products of polymers of ethylene
20 glycol and propylene glycol; and block copolymers of acrylamide and acrylic acid.

Typically the polymer will be water soluble. The polymer is preferably of very low toxicity in vivo and in
25 vitro, since many applications involve administration to man or animals and/or exposure to cells in cell culture systems. The polymer length is not restricted in this invention, since it will depend on the principal use of the product (e.g. short lengths may be best for coupling two
30 proteins together and longer polymers for improving plasma half life). With each application optimum polymer length must be selected either empirically or by reference to the desired application. The polymer's own inherent hydrophobicity is also important in some settings and will
35 have to be evaluated on a case-by-case basis.

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The reactive groups G are terminal hydroxyl groups. Depending upon the selected polymer such groups G may already be available for reaction or it may be necessary to introduce groups G by conventional techniques in a preliminary step. Generally groups G will be groups bonded to carbon atoms of the polymer. The groups G must be capable of reacting with the group X of the compounds of formula (I). The groups G will be selected such that they ultimately permit formation of the desired non-immunogenic, non-antigenic, non-toxic, non-biodegradable direct covalent link between the polymer and target. Preferred groups G are primary ($-\text{CH}_2\text{OH}$) or secondary ($-\text{CHOH}$) alcoholic hydroxyls for instance primary hydroxyls as found at the termini of polyalkylene glycols such as in PEG. Such hydroxyls react with sulphonyl halides and other activating compounds of formula (I) to form activating sulphonate ester moieties.

The use of capping groups C is an important feature of the reaction since this embodiment of the invention has special advantages discussed further below. The function of the capping groups C is to protect one or more reactive groups G of the polymer molecule from nucleophilic attack while leaving the activated polymer product susceptible to nucleophilic attack only at the carbon or equivalent atom adjacent to the groups $-\text{AM}$. C therefore must lack the properties of $-\text{AM}$ and should preferably be an inert group with respect to reactivity with other molecules and should be of low toxicity. Suitable groups C are well known to those skilled in the art as are techniques for introducing such groups. A preferred group C is methyl.

Production of the polymers for use in the process of the invention may be achieved by conventional techniques although most materials will be obtained commercially, possibly then being modified to introduce the desired reactive groups G and/or capping groups C as mentioned

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above.

A preferred polymer for use in accordance with the present invention is a PEG polymer $\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_x\text{-H}$ where x is large enough for the molecular weight to be in 150($x=3$), 194($x=4$), 238($x=5$), 282($x=6$) or above, for instance about 500, 1000, 2000, 3000, 4000, 5000, 6000 or 10000 which has two reactive groups G, i.e. the hydroxyl groups. The size of the polymer actually used will be selected according to the desired properties of the end product.

Another preferred polymer is MPEG (i.e. the polymer $\text{MeO}-(\text{CH}_2\text{CH}_2\text{O})_x\text{-H}$ which has one capping group C, i.e. the methyl group and one reactive group G, i.e. the hydroxyl group) where x is 3, 4, 5 or 6 or is large enough to provide molecular weights of, for instance, about 500, 1000, 2000, 3000, 4000, 5000, 6000 or 10000. Branched PEGs can also be used.

Since conventional polymerisation techniques generally produce materials which are mixtures, in certain cases some purification may be required before the polymer is used in the process of the invention, for example, some preparations of MPEG are significantly contaminated with PEG. This would lead to the production of biactivated PEG and crosslinking on subsequent use and needs to be avoided for many applications. Such contaminated material can be identified on the basis of its size distribution (polymerisation occurs at both ends of the molecule for PEG hence the contaminant has circa twice the molecular weight). Size fractionation should therefore be used to remove this PEG from MPEG preparations prior to activation: gel permeation chromatography and other appropriate methods may be used. For example, the divalent PEG can be separated from the bulk MPEG using vesicle chromatography [Selisko et al (1993) J. Chromatogr. 641, 71-79].

35 TARGET MATERIALS

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Suitable target materials to which polymer can be attached in accordance with the present invention are all materials having biological activity which are useful in, for instance diagnosis or therapy and which are all well-known to those skilled in the art. They all contain at least one reactive group (hereinafter referred to as groups "N") containing an atom which is capable of mounting a nucleophilic attack on the carbon or equivalent atom of the polymer adjacent to the group AM. Examples of the reactive group include primary and secondary amino groups, thiol groups and aromatic hydroxy groups.

More specifically, potential targets include proteins, peptides, amino acids and their derivatives such as: antibodies and fragments thereof; cytokines and derivatives or fragments thereof, for example, the interleukins (IL) and especially the IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10 and IL-11 subtypes thereof; colony stimulating factors, for example granulocyte-macrophage colony stimulating factor, granulocyte-colony stimulating factor (alpha and beta forms) and macrophage-colony stimulating factor (also known as CSF-1); haemopoietins, for example erythropoietin, haemopoietin-alpha and kit-ligand (also known as stem cell factor or Steel factor); interferons (IFNS), for example IFNalpha, IFNbeta and IFNgamma; growth factors and bifunctional growth modulators, for example epidermal growth factor, platelet derived growth factor, transforming growth factor (alpha and beta forms), amphiregulin, somatomedin-C, bone growth factor, fibroblast growth factors, insulin-like growth factors, heparin binding growth factors and tumour growth factors; differentiation factors and the like, for example macrophage differentiating factor, differentiation inducing factor (DIF) and leukaemia inhibitory factor; activating factors, for example platelet activating factor and macrophage activation factor; coagulation factors such as

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fibrinolytic/anticoagulant agents including heparin and proteases and their pro-factors, for example clotting factors VII, VIII, IX, X, XI and XII, antithrombin III, protein C, protein S, streptokinase, urokinase, 5 prourokinase, tissue plasminogen activator, fibrinogen and hirudin; peptide hormones, for example insulin and calcitonin; enzymes such as superoxide dismutase, glucocerebrosidase, asparaginase and adenosine deaminase; vaccines, for example hepatitis-B vaccine, malaria vaccine, 10 melanoma vaccine and HIV-1 vaccine; transcription factors and transcriptional modulators; carbohydrates, glycosoaminoglycans, glycoproteins and polysaccharides; lipids, for example phosphatidyl-ethanolamine, phosphatidylserine and derivatives thereof; sphingosine; 15 steroids such as cholesterol and derivatives thereof; nucleotides, nucleosides, heterocyclic bases, DNA, RNA, synthetic and non-synthetic oligonucleotides including those with nuclease resistant backbones; vitamins; antibiotics; bacteristatic and bactericidal agents; 20 antifungal, anthelmintic and other agents effective against infective agents including unicellular pathogens; small effector molecules such as noradrenalin, alpha adrenergic receptor ligands, dopamine receptor ligands, histamine receptor ligands, GABA/benzodiazepine receptor 25 ligands, serotonin receptor ligands, leukotrienes and tri-iodothyronine; cytotoxic agents such as doxorubicin, methotrexate and derivatives thereof.

The target molecules may also be part of larger multi-molecular structures. These include cells or parts 30 thereof, for instance erythrocytes, erythrocyte "ghosts" and leukocytes, liposomes such as multilamellar vesicles and unilamellar vesicles, micelles and micelle-like structures, and aggregates, microemulsions, coacervates, emulsions, suspensions of the foregoing.

35 It will be appreciated that when the target molecules are part of such structures there will generally be many

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target molecules in each structure; treatment according to the invention will therefore produce a structure bearing many polymer moieties. Where the polymers are bi- or multi-valent, reaction with a multimolecular target may result in
5 intermolecular cross-linking by the polymer between molecules of the same target structure and/or between molecules of different target structures as well as intramolecular bonding of the polymer to more than one position on the same molecule of a target structure.

10 It is evident from the inventors' investigation that much of the loss of biological activity frequently observed with prior polymer coupling methods is due to inappropriate coupling reactions (i.e. reactions taking place at sites which disable the target's biological activity), coupling
15 conditions and/or contaminating toxic material reducing responses in bioassays. The present process provides a means of generating adducts of polymer and target with highly conserved biological activity for the majority of molecules, or even enhanced biological activity. In the
20 event of unusually substantial loss of biological activity, a search must be made using fractionated material with different degrees of substitution and appropriate mapping (e.g. peptide mapping) to identify the group or groups N the modification of which by polymer is responsible for
25 lost activity. The situation can then be remedied either by modifying the group N to abrogate coupling of polymer at that site or by shielding N with an appropriate non-covalent bond (examples include hydrogen bonding of base pairs for DNA and protein/protein binding like receptor
30 ligand interactions). Two alternative strategies are available depending on the nature of the critical N group, in particular whether there are significant differences in the susceptibility of the group compared to the other such groups (either being more or less susceptible) to coupling
35 to the polymer. If the group N is less susceptible the coupling conditions (usually the coupling ratio) can be

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altered so that the critical group N is rarely modified. If it is more susceptible, a two step procedure where short polymer molecules are first linked to more accessible (susceptible) sites (i.e. including the group(s) N) then
5 long polymer molecules are linked to the remaining sites may prove acceptable, the outcome depending on the nature of the problem at the N site with respect to biological activity.

Targets lacking a reactive group may be modified so
10 as to create one or more reactive groups; this is within the ability of those skilled in the art and can be achieved by well-known techniques.

Some target structures (e.g. RNA and single stranded DNA) pose special problems because they provide too many
15 "N" groups to which the polymer would attach in a standard reaction. There the groups N may be temporarily protected by involvement in an appropriate conformation precluding the nucleophilic attack, as for example in the hydrogen bonding associated with base pairing of DNA (see below).

20 An embodiment of the present process involves site-specific modification of DNA, RNA and synthetic oligonucleotide targets (or of any molecule containing an amino or other reactive group which can participate in interactions such as hydrogen bonding with another molecule
25 or compound) by precluding the nucleophilic attack on the activated polymer species by reactive groups on the target. The bases adenine (A), cytosine (C) and guanine (G) [but not uracil (U) or thymine (T)] provide suitable targets in DNA, RNA and synthetic oligonucleotides for modification
30 with polymer moieties according to the invention and thus these are special targets with the problem that there are too many available reactive groups to which the polymer can be attached. As shown below, single stranded DNA is rapidly and very heavily modified by activated PEG (TMPEG).

35 By using various restriction fragment DNA cleavage sites as a model system, the present inventors have shown

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that selected bases A, C or G can be modified by the expedient of leaving short stretches (e.g. 2-4 bases) single stranded. Adenine bases appear to be the most susceptible to such modification. Blunt ended double stranded DNA is not readily coupled under the conditions described, indicating that hydrogen bonding between base pairs is sufficient to preclude interaction of the amino groups of A, C and G bases with the activated polymers.

Site-specific DNA modification by polymer can be achieved by the expedient of including one or more A, C or G bases in a short single stranded section of DNA by appropriate restriction enzyme digestion or by hybridising oligonucleotides of dissimilar lengths with the DNA to protect bases which are not to be modified or by exploiting the natural strand asymmetry of polymerase chain reaction products which have a one base-pair overhang, or by exploiting localised regions of single strandedness achieved by natural or artificial localised melting of the double helix.

THE PROCESS

The process of the present invention relies upon conducting steps (a) and (b) in accordance with certain constraints as defined above. In particular, step (a) must be conducted using dry polymer, dry solvents and with water being excluded from the reaction vessel. Dryness of the polymer may be determined by benzene distillation as described below. If the polymer is not sufficiently dry, interfering side reactions occur making it difficult to obtain economical amounts of the final adduct. Likewise, wet solvents and ingress of water during the reaction will also cause interfering side reactions. Suitably dry solvents are obtained using molecular sieves (0.3nm) as described below. Exclusion of water from the reaction vessel can be achieved by conventional techniques.

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Benzene distillation

The principal exploited here is that the water-benzene azeotrope has a boiling point of 65°C and that after water has been removed from a sample, the distilling temperature rises to that of pure benzene (i.e 79 to 80°C). In addition, the water-benzene azeotrope is cloudy and benzene is clear so that the change from cloudy to clear distillate confirms that drying is complete. If a suitably dry sample is heated in benzene the distillation temperature will be that of pure benzene and the distillate will be clear from the first.

0.3 nm Molecular sieves

Solvents may be dried by any conventional method provided that they are dried to the extent obtainable by use of 0.3 nm molecular sieves. Thus, for instance, when 0.3 nm molecular sieves are used an amount of molecular sieve is added to and left in contact with the solvent for several hours (preferably about 10 hours or more) and at about room temperature (ca 20°C), the amount of molecular sieves used suitably represents a twenty fold excess over the recommended amount to adsorb the anticipated water content in the solvent, according to the manufacturer's instructions, for instance about 100g of molecular sieve per litre of substantially dry solvent.

It is also a requirement that the solvent should be inert to the reagents and the activated polymer. Suitable inert solvents include halogenated alkanes such as dichloromethane and other organic solvents with low hydrophilicity and low hygroscopicity; such solvents are well known to those skilled in the art. Halogenated alkanes, especially dichloromethane, are preferred.

The activated polymer is preferably used directly following its production in accordance with step (a) as this affords the minimum opportunity for degradation.

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leading to formation of other reactive species which will interfere with subsequent reactions. If the product is to be stored prior to use, for instance when formed in bulk for use in smaller quantities at a later date, it must be
5 stored so as to avoid hydrolysis, i.e. in the presence of a more hygroscopic desiccant. Sulphonyl halides are highly hygroscopic and will dry many common desiccants such as silica gel and thus become hydrolysed. The preferred desiccant for drying and storage of the activated polymer
10 is phosphorus pentoxide.

The reaction of the activated polymer with the target material in step (b) takes place in a non-denaturing medium; for many biological materials this will of necessity be an aqueous medium. There are also many target
15 materials which are stable under non-aqueous conditions and for these it is quite acceptable to conduct step (b) in organic solvents which are inert to the reagents. Ultimately the choice of reaction medium will be dependant on the stability of the target material and final adduct in
20 that medium. Selection of pH, salt concentration, protein concentration and other requirements for stability will be determined on a case-by-case basis. Those skilled in the art will have no difficulty with this. The same applies in relation to determination of non-denaturing temperatures.

25 When the process of the invention involves the use of a solid support coupled to the activated polymer, the reactions involved in coupling the activating moiety to the solid support will also be conducted under dry conditions, i.e. dry reagents as determined by benzene distillation and
30 dry solvents as determined using molecular sieves of 0.3 nm, will be employed whenever sulphonyl halide groups are present.

It is preferred that the reaction of step (a) is conducted in the presence of a base such as a tertiary
35 amine, preferred bases include those which are easily removed by washing, such as pyridine, and volatile bases

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which may be removed by evaporation. At present the preferred base is pyridine. However certain bases, especially pyridine, may react deleteriously with the target material and these should be removed prior to step 5 (b). This may be achieved by conventional means.

It is preferred that the reagents for step (a) are mixed at reduced temperature, preferably at 0°C for instance by chilling with ice and that the reaction mixture is then allowed to warm to ambient temperature, for 10 instance about 20°C.

These preferred features may be adopted in any combination but most preferably all of these preferred features are adopted.

When the polymer of formula (II) has more than one 15 group G and/or one or more capping groups C, it has been found that contamination with related products of formula (II')



20 wherein C, POL and G are as defined above and c' and g' are zero or positive numbers such that $c' + g' = c + g$ but g' is different to g , can lead to undesirable inhibition of the intended reaction and formation of unwanted by-products 25 which therefore complicates the purification and recovery of the eventual adduct. In the case where the polymer of formula (III) is a polyalkylene oxide with one capping group (eg MPEG), c is 1 and g is 1. Contaminants of formula (II') in which c' is 2 and g' is 0 will be unreactive and 30 thus not detrimental but contaminants in which c' is 0 and g' is 2 will cause cross-linking of target materials; preferably such contaminants are avoided in production of the compounds of formula (II) or are removed prior to use of the compound in step (a) of the present process. When 35 the polymer of formula (II) is a material in which $c' + g' > 2$ there are many possible contaminants; again those

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wherein g' is 0 are unreactive and of little concern whereas those wherein $g' > 1$ and $g' > g$, especially those wherein $g' > g$, cause unwanted side reactions and are preferably removed from the reagent before use.

5 Whilst the reaction of step (b) will proceed in the presence of large amounts of such contaminants, it is preferred that the amount of all compounds of formula (II') present in the compound of formula (II) is less than 35%,
10 for instance less than 25%, more preferably less than 20%,
15 for instance, 15, 10 or 5% and yet more preferably below 1% by weight based on the total weight of the compound of formula (II) and all compounds of formula (II'). This may be achieved by purifying the starting materials used in forming the polymer, controlling the polymerisation and
15 subsequent process steps required to form the compound of formula (II) and/or by purifying the compound of formula (II) before use in the process of the invention.

 In a preferred aspect of the invention the compound of formula (II) is MPEG in which POL is a divalent residue
20 $-O-(CH_2CH_2O)_x-$ (for instance the residue of PEG 5000), c is 1 and C is methyl and g is 1 and G is hydroxyl. This material is produced by polymerisation from methoxyethanol and ethylene glycol and tends to contain a large proportion of material where polymerisation has initiated from
25 ethylene glycol rather than the methoxyethanol such that chain extension occurs in both directions and a PEG product of approximately twice the molecular weight of the MPEG is present as a major contaminant; this may readily be removed by gel permeation chromatography at an appropriate
30 molecular weight cut-off.

 Purity can be checked by nmr and chromatography. Contamination by materials where $g' > g$ is particularly to be avoided when 1:1 target:polymer adducts are to be produced, since the presence of such contaminants will lead
35 to the production of aggregated materials.

 When the reaction of target and activated polymer is

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to be performed at a solid:liquid interface, the activated polymer may, in one variant of the process, be linked to a solid support after reacting a compound of formula (I) having a suitable linking group with the polymer. The linking reaction may be effected as described above in connection with the production of compounds of formula (I) linked to a solid support. Thus, for instance, a tetrafluoro-4-amino-azobenzene-4'-sulphonyl derivative of the polymer is reacted with a solid support bearing aldehyde groups (for instance siliconised glass treated with glutaraldehyde) in the presence of a non-nucleophilic base, such as lithium isopropylamide, followed by borohydride reduction of the potentially labile double bond.

It is particularly preferred that the process of the invention is conducted using tresyl chloride as the compound of formula (I) and MPEG, for instance MPEG 5000, as the polymer of formula (II). For MPEG of molecular weight up to 5000 or 6000, purity can be checked by nmr but for higher molecular weight material the methyl signal is swamped and chromatographic techniques must be adopted. These are well known to those skilled in the art.

In this preferred aspect, the product of formula (III) is TMPEG. Ideally this is used immediately but, if not, storage of TMPEG is critical and activity is not well maintained unless it is properly stored. When desiccated over phosphorus pentoxide and stored at 4°C, activity is maintained for at least 1 year. Degradation of TMPEG (either during production or on storage) is detectable on the basis of a change in the FPLC profiles of exposed peptide targets (see Example 1). It produces less substitution of the target at a given molar ratio (a loss of resolution and right shift of the elution profile accompanies the former if FPLC loads are not corrected for the amount of PEG loaded). Substantial aging of TMPEG prepared as previously described is documented in Figure 8

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in WO90/04606. Since degradation produces coproduct, degraded TMPEG preparations are also more acidic.

PEG itself is known to oxidise on storage to a brown discoloured product. The TMPEG preparation process removes
5 the antioxidants present in commercially supplied PEG. Oxidation has been observed in several stored TMPEG preparations and it is recommended that, if prolonged storage is anticipated, an appropriate antioxidant should be included. This new method for preparing and storing the
10 TMPEG has significant advantages over previously described methods in terms of the activity, reproducibility between preparations and stability of individual preparations.

In an especially preferred aspect the present invention provides a process comprising the steps of

15

(a) reacting dry MPEG (as determined by benzene distillation) containing less than 10% by weight of PEG (as determined by nmr and/or chromatography) with tresyl chloride in dry
20 dichloromethane (as determined using 0.3 nm molecular sieves) in the presence of pyridine in a reaction vessel from which water is excluded, so as to form TMPEG, recovering the TMPEG so formed and either storing it over
25 phosphorus pentoxide or using it directly in step (b),

(b) reacting the TMPEG so formed, optionally after storage, with a target material such as
30 erythropoietin (EPO) in aqueous medium and about 20°C and

(c) recovering the Target:MPEG adduct (eg EPO:MPEG) so formed.

35

More preferably the molar ratio of tresyl chloride to

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MPEG is 2:1 or more, most preferably about 2.5:1.

The process of the present invention may be supplemented by one or more separation and/or purification steps at any stage as necessary or desirable. Separation and purification may be achieved by appropriate techniques well known in the art selected having regard to the need to maintain the activity of the reagents and products and to avoid use of toxic or interfering materials. In some cases, for instance where the polymer is a polyalkylene glycol, aqueous biphasic polyalkylene glycol solutions may be used to effect separation and/or purification of the activated polymer and/or polymer:target adduct.

THE PRODUCTS

15

The present invention further provides polymer:target adducts obtainable by the process of the invention other than the known polymer:target adducts having polymer and target moieties directly linked by stable, non biodegradable covalent bonds which are described in the references discussed above.

The invention also extends to such products for use in therapeutic and diagnostic methods of treatment of the human or animal body and to the use of such products in the manufacture of medicaments for use in therapeutic and diagnostic methods of treatment of the human or animal body and to pharmaceutical compositions comprising products of the invention together with pharmaceutically acceptable diluents or carriers.

Many of the prior methods of coupling polymers to targets are unsuitable for coupling more than one target molecule to each polymer molecule since most methods use polymers having only one group capable of reacting with the target. In contrast, the process of the present invention enables the production of constructs such as the following which form particular embodiments of the present invention:

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1. Liposomes bearing linear polymeric linking groups bound to the surface of the liposome at one terminal of the polymer and bearing a receptor ligand, receptor molecule, antibody, antigen or other molecule of therapeutic or
5 diagnostic interest at the other terminal.

2. Constructs where a plurality of identical target molecules are linked to a single polymer moiety; for instance a linear polymer having two reactive termini is
10 linked to two target molecules to enhance the biological activity thereof or a branched polymer having three or more reactive termini or a polymer having reactive pendant groups is linked to three or more target molecules.

15 3. Constructs as in 2. above wherein the target molecules are different; such constructs may be intended to have synergistic effect when the target molecules interact with each other or with other substrates.

20 4. Constructs wherein the polymer moiety is linked to a target molecule at two or more positions either by reaction of both termini of a linear polymer with different sites on the target molecule or by reaction of pendant reactive groups, or the termini of a branched polymer, at
25 two, three or more sites on the target molecule.

The products of the present invention preferably comprise any one of the polymer materials set out above and any one or more of the target materials set out above.
30 Particularly preferred products of the invention are adducts of a polyalkylene glycol, especially polyethylene glycol and polypropylene glycol with the former being most preferred, with any one or more of the target materials set out above, with erythropoietin (EPO) being particularly
35 preferred.

The production of adducts wherein two or more

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different target species are coupled to a multivalent polymer presents special difficulties which can be overcome by the process of the present invention. For this situation an appropriate procedure for producing a product comprising
5 two different targets involves the following steps:

1. A first target, Target (A), is exposed to activated polymer (AM-Polymer-AM) with the latter in gross excess such that essentially only 1:1 Target(A):polymer
10 adducts are formed and 2:1 or higher Target(A):polymer adduct formation is minimised. This can be followed by FPLC or other appropriate methods to separate and, if necessary, purify the adduct, provided that this is sufficiently rapid to avoid hydrolysis of the remaining activating moieties.
15 If the target has many derivatisable groups, it may be necessary to fine-tune the coupling ratio, reaction time and/or pH, so that 2:1 or lower Target(A):polymer adducts are not formed or, if formed are discarded. Alternatively a mixture of monovalent and multivalent activated polymer
20 species may be used in a ratio selected such that it is statistically likely that only one of the polymer molecules attached to any target molecule will be the multivalent form (targets which are by chance only modified with the univalent form will be wasted).

25

2. Either:

a) the Target(A):polymer adduct is first
30 separated from the AM-polymer-AM then reacted with the second target, Target(B), preferably at equimolar ratio or, if this reacts too slowly, with the adduct in excess; or

35

b) the reaction product of step 1 is reacted

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with Target(B), preferably at a molar ratio of 1:2 with respect to the amount of activating moieties remaining in order to reduce the formation of Target(B):polymer:Target(B) constructs. This is much simpler than (a) but more wasteful in terms of Target(B) which is therefore selected as the cheaper of the targets.

10

After a sufficient reaction time (derived empirically), the excess -AM is neutralised with a monovalent neutralising agent e.g. with glycine, and the products are purified to discriminate the combinatorial possibilities. This may be possible on the basis of molecular weight if target(A) and target(B) differ significantly in size or via differential labelling of the targets. Alternately, since the major contaminant will be Target(B):polymer:Target(B) at this stage, an affinity purification method, with affinity ligand to Target(A), should enrich for hybrids. Similar measures may be adopted to construct more complex adducts.

A particular advantage of the present invention is that the process enables the coupling of polymer moieties to oligonucleotides and nucleic acids (DNA and RNA) at defined positions. EP-A-O 292 128 (Seeger 1988) deals with "improved DNA probes" and uses PEG, amongst other molecules, to link DNA to reporter groups for hybridisation. However the PEG is linked to the 5'-phosphate of the DNA and there is no indication of a method which would link the PEG without leaving a coupling moiety. This ability to modify DNA, RNA and synthetic oligonucleotides with polymer will influence the solubility and alter biodistribution and has applications in both the in vitro and therapeutic or diagnostic (in vivo) use of nucleotides.

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Further improvements offered by the invention include the ability to maximise the biological activity retention and/or to increase the activity of the target molecule, minimise the toxicity of the product, minimise the reaction
5 time at physiological pH, reduce contamination of the product and improve the stability of the activated polymer.

The invention will be illustrated with reference to the accompanying figures of the drawings in which:

10 Fig.1 shows the results of separations of MPEG 5000 obtained commercially.

Fig.2 is a plot of age of TMPEG used and % of gm-CSF left unmodified by reaction with aged TMPEG produced according
15 to the invention.

Fig.3 is a plot of the relative activities of PEGylated gm-CSF prepared by the process of the invention and by a previously described process.

20

Fig.4 plots the activity of EPO and PEG-EPO.

Fig.5 is a gel showing DNA fragments variously treated with TMPEG, MPEG or buffer.

25

Fig.6 shows log concentrations of gm-CSF and various PEG-gmCSF adducts in blood and tissue of mice.

Fig.7 shows the results of fractionation of gm-CSF and
30 PEG-gm-CSF before and after ageing.

Fig.8 shows FPLC profiles for EPO PEGylated with various molar ratios of TMPEG:lysine.

35 Fig.9 shows biological activity of unmodified and PEGylated EPO.

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The present invention will now be illustrated by the following Examples which are not intended to limit the scope of the invention in any way:

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EXAMPLESExamples 1a and b and Comparative Examples 1a and b(a) Purification of MPEG

5 MPEG-5000 (Union Carbide) was subjected to permeation gel chromatography on Superose 12 (Fig. 1a). The material has a clearly biphasic size distribution with an estimated 22.9% high molecular weight contaminant (9280Da) and a low molecular weight component (5460Da). The good agreement
10 between proportions of the two peak areas and the NMR estimate of PEG versus MPEG in the same preparation suggest that all the PEG is accounted for by the high molecular weight species. To confirm this and to devise an appropriate purification procedure, vesicle chromatography
15 [Ehwald (1991)] was used. Analytical and preparative scale preparations gave different efficiencies of separation. Analytical scale separation was performed using vesicular packing material P1(5) equilibrated in 0.05M NaH_2PO_4 buffer pH 5.5 (column bed volume 22 ml). The MPEG preparation
20 (Union Carbide Mw 5000), 0.5ml of a 20mg/ml solution in PBS was loaded onto the column. The column was eluted with PBS using a flow rate of 0.08 ml/min and 0.5 ml fractions were collected. The elution profile is shown in Fig.1b; circa 8% of the material was eluted in the excluded fraction (high
25 molecular weight) and 92% in the permeable fraction (low molecular weight fraction). In preparative scale (using 6.5L bed volume and 15g/500ml MPEG in 0.01M NaH_2PO_4 pH 5.5 and an elution rate of 20.5 ml/min) the excluded fraction (PEG) was 11.2 % (Fig.1c). Rechromatography on Superose 12
30 of the two pooled fractions (shaded and hatched areas of Fig.1c) taken from the preparative scale fractionation on Superose 12 was used to determine the extent to which the two materials were separated (Fig. 1d, squares - permeable fraction, crosses - excluded fraction). NMR analysis of the
35 permeable fraction gave an estimated ratio of PEG:MPEG of 11:89 mole:mole, which is in good agreement with the area

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under the curve estimate of the residual high molecular weight contaminant in the permeable fraction on the elution profile on Superose 12 (Fig.1d, squares). NMR analysis could not be performed on the excluded fraction because of its much higher molecular weight. Further rounds of vesicle chromatography, at analytical scale, gave a comparable reduction in the high molecular weight material (the permeable fraction yielded a smaller "excluded" peak on rechromatography, circa 4% in the high molecular weight excluded fraction, using a second round of vesicle chromatography, Fig. 1e).

(b) Preparation of TMPEG

Tresyl chloride was used when freshly prepared or after storage in airtight ampoules. MPEG-5000 (Mr 5000, 18g, 3.5 mmol) was dried with benzene (B.P. 79-80°C) by distillation of the water-benzene azeotrope and then the benzene. The water-free MPEG was then immediately dissolved in dichloromethane (45 ml, ANALAR, previously dried over molecular sieve 3Å, 100 g per litre of solvent, overnight at room temperature). Activation of MPEG-5000 with tresyl chloride was carried out at a molar ratio of 2.5:1 (tresyl chloride to available hydroxyl groups in MPEG). The MPEG/dichloromethane mixture was then cooled on ice (to circa 3°C) and pyridine (1 ml, 12.4 mmol) followed by tresyl chloride (1 ml, 9 mmol), both precooled on ice were added dropwise with constant stirring using a magnetic stirrer. The reaction was allowed to continue (for 2 hr) at room temperature with constant stirring before the removal of dichloromethane by evaporation under reduced pressure. The solid obtained (TMPEG) was redissolved in methanol-hydrochloric acid mixture (1000:0.3 v/v) and allowed to precipitate overnight at reduced temperature (-20°C). The precipitate was recovered by centrifugation (at 1100 x g, for 10 minutes) at reduced temperature (0°C) and the supernatant was spectrophotometrically checked for pyridine content (λ_{max} 255nm). This procedure was repeated until no

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pyridine could be detected, usually 12 to 15 washes. Finally, the pyridine-free precipitate was washed in methanol only, preferably at least twice, and then the methanol was removed by evaporation under reduced pressure and the TMPEG was finally dried by freezing in liquid nitrogen and lyophilising under reduced pressure. The hydroxyl content of the white solid as determined by ¹H nmr obtained was undetectable (4.56ppm). Therefore, approximately 100 % of hydroxyl groups in the MPEG were transformed into tresyl esters.

The TMPEG so produced was desiccated over phosphorus pentoxide and stored at 4°C.

(c) Coupling to GM-CSF

The activity of TMPEG samples was monitored by exposure of the samples to gm-CSF at molar ratios of 305:1 (Example 1a and Comparative Example 1a) or 75:1 (Example 1b and Comparative Example 1b) in a standard coupling reaction and estimating the percent of the preparation remaining unmodified after the reaction as shown in Fig.2: the relative activities of TMPEG preparations obtained as described above (Examples 1a and b) and of TMPEG made by a prior art process (Comparative Examples 1a and b) is plotted against the storage period. TMPEG made by the process of the invention and used at 305:1 (squares), throughout the observation period, gave <10% residual unmodified gm-CSF. The equivalent result when using the same material at 75:1 (diamonds) was circa 20% unmodified material. With both preparations there was no evidence of a trend of worsening activity with storage. In contrast preparations made from MPEG as obtained from the manufacturer by a previously described method (WO90/04606, Example 1) (triangles; downwards triangles 305:1 and upwards triangles 75:1) showed: (1) lower mean activity (reflected in higher mean residual unmodified material); (2) a very broad scatter of reactivities; (3) 3 samples completely inactive at 75:1 and 2 inactive at 305:1 after

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only 5-12 weeks of storage. At 305:1 the process of the invention consistently produced 92-95% modification, irrespective of the age of the TMPEG and using a coupling ratio of 75:1, circa 80% of the gm-CSF preparation was
5 modified.

The process described above (Example 1) has been repeated on a number of occasions with similar results.

10 The gm-CSF PEGylated using the process of Example 1 showed no significant loss of its growth stimulating activity as assessed by granulocyte-macrophage colony assay (Fig.3a) or by thymidine uptake (Fig. 3b). Dose response analyses are a relatively insensitive means for
15 demonstrating subtle differences in bioactivity and are also difficult to analyse after pooling because of the high contribution to errors of the variation in the response level between experiments. A better approach is to perform a correlation analysis, plotting the response obtained with
20 equivalent amounts of the modified and unmodified materials on an XY plot. Identical activity gives datum points randomly distributed about the line representing equal activity (the dashed lines). Reduced activity reveals itself as a curvilinear departure from the equal activity
25 line which converges towards the line at the extremities (if sufficiently broad a dose response range is examined such that the plateau of maximum stimulation is reached). In contrast, the presence of inhibitory material, with or without loss of intrinsic activity, produces a departure,
30 usually without convergence to the upper extremity. In the latter case (i.e. where there is no loss of intrinsic activity) failure to observe divergence at the lower extremity indicates that the inhibitor is masking activity only at relatively high inhibitor levels; divergence
35 throughout the plot indicates either an inhibitor with powerful effect at low dilution or combined inhibition and

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loss of activity.

Two assays for the growth stimulating activity of GM-CSF prepared by the methods of Example 1 (□ in Fig.3) and Comparative Example 1 (□ in Fig.3) show that the process of the invention gives better conservation of biological activity. Colony counts or 3H-thymidine uptake (normalised with respect to the maximum per experiment to avoid a contribution of between-experiment differences to the correlation) were plotted for comparable amounts of PEG-GM-CSF and GM-CSF over the dose range spanning the upper and lower asymptotes of the dose response curve (0.1 to 8 ng/ml for colony assays and 0.0006 to 1.0ng/ml for thymidine uptake assays). Datum points are means of colony counts in triplicate dishes for three independent experiments (Fig. 3a) or of two independent thymidine uptake experiments (Fig 3b). The results in the thymidine uptake demonstrate no significant loss of activity with the process of Example 1 and loss of activity without significant inhibitory material for the process of Comparative Example 1 (TMPEG in equivalent doses was not inhibitory in this assay). The results for colony stimulating activity again show no significant loss of activity with process of the invention but indicate an additional inhibitory activity for this assay when the old method was used possibly accompanied by loss of bioactivity.

Example 2

a) Preparation of ditresyl PEG (TPEGT)

PEG-6000 (10.5 g, 1.75 mmol) was dissolved in benzene (30 ml) and dried as in Example 1 above. The dry PEG-6000 was then immediately dissolved in 25 ml of dichloromethane (dried as in Example 1), cooled on ice and pyridine (1 ml, 2.4 mmol) followed by tresyl chloride (1 ml, 9 mmol) were added dropwise. The mixture was left to react (two hours at room temperature) and the dichloromethane was evaporated as

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in Example 1. The solid was resuspended in methanol:hydrochloric acid and was treated as above.

b) Coupling to GM-CSF

5 To test that this material was bivalent and could couple target(s) at both ends of the PEG chain, this TPEGT was used to aggregate GM-CSF. ¹²⁵I-GM-CSF (1.4nmol/ml) was reacted with TPEGT (160mg/ml) for 2h at room temperature. The resulting aggregates were estimated by FPLC and
10 contained 95% of the total GM-CSF. Thus the bivalent AM-polymer-AM construct is demonstrated to be functional.

Example 3

Example 2 was repeated using TMPEG prepared from a
15 sample of MPEG containing 8% PEG as contaminant, and thus containing about 8% TPEGT. When used to aggregate GM-CSF, this material (160 mg/ml) produced an aggregate estimated by FPLC to contain only 20% of the total GM-CSF. This demonstrates that the higher proportion of conjugates seen
20 in Example 2 was related to the additional TPEGT and not merely due to non-specific aggregation. It should be noted that using a prior art process, the present inventors have shown [Malik et al (1992) Experimental Hematology, 20, 1028, fig. 4] that both the non-specific aggregated gm-CSF
25 in unPEGylated reactions and aggregates generated in PEGylation reactions (which include cross-linked products due to TPEGT) have an extremely high specific activity, as assessed by thymidine incorporation, with respect to monomeric gm-CSF). Thus it is anticipated that the bivalent
30 2-polymer construct generated in Examples 2 and 3 would have a higher specific activity than monomeric unmodified gm-CSF.

Example 4 and Comparative Examples 2,3 and 4

35 To document the better conservation of biological activity, direct comparisons were made with three of the

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widely used current methods, using erythropoietin as the target peptide. It is an important prerequisite in experiments of this type to perform preliminary experiments (using at least two independent assays of bioactivity and
5 some measure of the degree of modification) to select the optimum coupling ratio and duration of coupling reaction for maximum retention of biological activity. It is important to record the degree of modification since this may vary between the methods being analysed and only
10 materials substituted to approximately the same extent should be compared for retention of activity.

Serial dilutions of TMPEG produced as in Example 1 were mixed with the same amount of erythropoietin and the resultant PEG-erythropoietin adducts were assessed first by
15 FPLC for the degree of modification and also in the thymidine uptake assay [Malik et al., (1992) Experimental Hematology, 20: 1028] for a preliminary estimate of activity. Based on the latter a smaller range of activities were analysed in colony assays using the methyl cellulose
20 assay system of Ash et al., Blood, 58:309-316 (1981), Knusli, C. et al. (1992) Br. J. Haematol. 82(4): 654.

Commercially available activated PEGs (Sigma Limited) were used to PEGylate recombinant human erythropoietin (Cilag Ltd.) by the cyanuric chloride (Comparative Example
25 2), succinimidyl succinate (Comparative Example 3) and phenylchloroformate (MPEG-p-nitrophenylcarbonate) methods (Comparative Example 4). TMPEG, prepared as described above, or the alternate activated PEGs, were incubated (for
2 h) with erythropoietin (100 µg/ml total protein, i.e.
30 carrier plus erythropoietin) in coupling buffer (PBS) at room temperature in a rotary mixer, at an activated-PEG:lysine molar ratio of 75:1. Conditions were standardised so that differences in pH and duration of the coupling reaction could not account for differences in the
35 residual bioactivity. Representative reactions at the same molar ratios were checked by FPLC and found to be ≥85%

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modified. Modified material was assayed in methylcellulose cultures of normal human bone marrow progenitor cells essentially by the method of Ash et al (1981) Blood, 58 309-316, but substituting recombinant human GM-CSF for leucocyte conditioned medium. Erythroid bursts were scored microscopically on encoded, encrypted dishes on day 14-16 of culture. The results for the optimised TMPEG-derived adducts and for products of the other coupling reactions having the same coupling ratio are shown in Fig.4 as means of triplicate culture dishes except in Panel A where the results are means \pm SEM for 3 independent experiments. Fig 4a shows the excellent conservation of biological activity achieved with EPO PEGylated with the TMPEG (filled circles) compared with unmodified EPO (open circles). Datum points are superimposed except at 4U/ml (33.6 ng/ml) erythropoietin. Similar effects observed in CFUgm assays were found to be due to a modest inhibitory effect of TMPEG in the assay system.

Fig. 4b shows a complete loss of detectable activity when the cyanuric chloride method (filled circles) was used compared with PEG-EPO prepared according to Example 1 (open circles) and a high dose inhibition of endogenously stimulated erythroid colonies. This indicates either the presence of a profoundly inhibitory substance in the reaction mixture or loss of all intrinsic erythropoietic activity in conjunction with some inhibitory material. Lack of even a slight increment in colony formation at low doses and the shallow dose related inhibition of endogenously stimulated colonies at high concentrations favours the latter interpretation. The FPLC profile for this material also differed somewhat from that obtained with the other three activated PEG preparations in that there was a higher proportion of material eluting with the void volume, indicating the presence of more aggregates. This suggests that crosslinking of target molecules may be occurring (as has previously been observed for this agent [Leonard

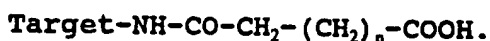
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(1984)]).

Fig. 4c shows a somewhat different picture for PEG-EPO prepared by the succinimidyl succinate method (filled circles), with detectable activity only at 4U/ml (33.6ng/ml) compared with PEG-EPO according to the invention (open circles). This type of shift in the dose-response curve is less likely to be due to inhibitor and probably represents over an order of magnitude loss in bioactivity. This is somewhat surprising because this method is claimed to have good conservation of biological activity [Katre (1987), Zalipsky (1992)]. However such claims have been made on the basis of short term assays. In the assay used here the erythropoietin needs to be available throughout the 14-16 day culture period. The cells in these cultures are also a rich source of esterases and the PEG-erythropoietin made by this method contains a potentially labile bond (see Table 1). In this and some related active ester methods, removal of the PEG leaves the molecule modified as follows:- Target-NH₂ forms



then esterase cleavage forms



This process might well influence bioactivity adversely since there is a charge conversion from a lysine amino group to a carboxyl group at the previously PEGylated site.

Fig. 4d shows PEG-erythropoietin made using monomethoxy PEG-p-nitrophenylcarbonate (filled circles) compared with unmodified (sham-treated) EPO (open circles). Here there are increments in colony number at low doses similar to that seen with the tresyl chloride activated PEG, but the high-dose inhibition of colony formation is

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much more evident, indicating the presence of inhibitory material.

Comparative Example 5

5

An attempt was made to make PEG-erythropoietin using the carbonyldiimidazole method under comparable conditions to those used above, but on this time scale and coupling ratio the degree of modification achieved was not
10 significant.

Example 5

A solution (50 μ l) of amphotericin (Fungizone) in
15 distilled water (20 mg/ml) was mixed with HEPES buffer (450 μ l, pH 9.0) containing TMPEG produced according to Example 1 (60 mg/ml). The mixture was then incubated at room temperature for 24 h. As a control, amphotericin was similarly treated but with MPEG substituted for TMPEG. To
20 demonstrate the covalent linkage of the TMPEG to the amphotericin, the partition coefficient of amphotericin in both samples was measured as follows: 100 μ l samples were mixed with 1 ml of top and 1 ml of bottom phases of a biphasic system containing 5% dextran T-500, 5% PEG-6000,
25 0.15 M sodium chloride and 0.01 M sodium phosphate pH 6.8. After shaking for 1 min. the system was left to settle for 20 min. and then 500 μ l from the top and bottom phases were removed to quantify amphotericin (by measuring the absorbance at 330 nm). The % material in the top (PEG-rich)
30 phase was: a) control $4.9 \pm 0.2\%$; b) MPEG control $4.1 \pm 0.4\%$; c) TMPEG (preparation 1) $92.8 \pm 0.1\%$; d) TMPEG (preparation 2) $92.7 \pm 0.2\%$.

Example 6

35

As an example of creation of an affinity ligand PEG-

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WGA (polyethyleneglycol modified wheat germ agglutinin) was constructed in exactly the same manner as used in Example 1 and used to bind rightside-out vesicles from human erythrocyte membranes thus altering their partition in a PEG-dextran aqueous two-phase system. Rightside-out vesicles exposed to phosphate buffered saline prior to partitioning showed the following distribution: $17.6 \pm 1.2\%$ to the top phase, $48.7 \pm 9.0\%$ to the interface and $33.6 \pm 8.6\%$ to the bottom phase (mean \pm SEM, $n=3$). Rightside-out vesicles exposed to the PEG-WGA ligand prior to partitioning showed the following distribution: $30.1 \pm 1.2\%$ to the top phase, $32.7 \pm 1.4\%$ to the interface and $7.2 \pm 0.6\%$ to the bottom phase. This change in partitioning can be exploited to remove rightside-out vesicles from a mixture with inside-out vesicles thus obtaining a pure preparation of inside-out vesicles from human erythrocyte membranes.

Example 7

20

Aliquots of human erythrocytes ($50\mu\text{l}$ containing 0.95×10^7 cells) were added to one of the following reagents ($500\mu\text{l}$):

a) sodium phosphate buffer (0.05M, pH 7.5) containing sodium chloride (0.125M) (PBS).

b) TMPEG freshly prepared as in Example 1 in PBS (36 mg/ml, equivalent to 1.9 ng/cell).

c) TMPEG (36 mg/ml) inactivated with lysine (Sigma Ltd. 4mg/ml) at 2.5:1 molar lysine:TMPEG for 2h at room temperature.

After 30 min incubation at 37°C cells were pelleted down and resuspended in PEG-rich top phase (1 ml) so that their PEGylation could be monitored in a PEG-dextran phase system. Cells incubated in PBS partitioned with $26.2 \pm 11.8\%$ (mean \pm SD) cells in the top phase. With TMPEG exposure of cells this increased to $72.1 \pm 10.7\%$, whereas

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with prior inactivation of TMPEG to prevent coupling to cells there was only $32.7 \pm 9.3\%$ in the top phase, indicating that covalent attachment of the PEG to the erythrocytes was responsible for the change in partitioning behaviour. The erythrocytes remained intact in the TMPEG-treated and control experiments indicating that the method does not disrupt the cell membrane.

10 Example 8 and Comparative Example 6

Liposomes were reacted with TMPEG prepared by the process of WO90/04384 (Comparative Example 6) and the process of Example 1 (Example 8). The success of the PEGylation was followed in a PEG-dextran phase system by quantitating the liposome content of the top (PEG-rich) and bottom (dextran-rich) phases and at the interface. Because the TMPEG prepared by the process of Example 1 was known to be more active, only half the molar ratio was used. Despite this, a much higher degree of substitution of the liposome was achieved. This greater reactivity has the advantage that the liposomal preparation does not have to be exposed to so much activated PEG (the level of the latter is limited by its effect on liposomal stability and/or aggregation). 100 nm diameter unilamellar vesicles of bovine spinal cord phosphatidylserine (PS) were prepared by extruding lipid dispersions of PS (10 mg/ml in HEPES buffer pH9), spiked with ^3H -dipalmitoylphosphatidylcholine through 100 nm polycarbonate filters ten times using an extruder device (Lipex Biomembranes, Canada). Vesicles (300 μl) were incubated with TMPEG (39 mg, molar ratio TMPEG:PS at outer surface 4:1 or 19.5 mg, molar ratio 2:1) in HEPES buffer (60 μl) for 18 hrs at room temperature. As a control MPEG was substituted for TMPEG. To demonstrate the covalent attachment of PEG to the outer surface of the vesicles the distribution of the vesicle in a two-phase system was measured as follows: samples (20 μl) were mixed with top

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phase (1 ml) and bottom phase (1 ml) of a biphasic system containing dextran T-500 (5%) and PEG 6000 (5%), sodium chloride (0.15M) and sodium phosphate buffer (0.01M) prepared at 25°C. After shaking for 1 min, triplicate
5 samples (50 μ l) were removed for total counts. After the phases had separated at 25°C for 20 min triplicate samples were taken from the top phase (50 μ l) and the bottom phase (20 μ l) for scintillation counting. Vesicle partitionings, calculated as the percentage of added vesicles that were
10 present in the top(T) and bottom(B) phases and at the interface (100-T-B) were as shown in Table 2.

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Table 2

5	TMPEG sample	TMPEG:PS Molar ratio	Partitioning (%) (mean of triplicate partitions)		
			Top	Interface	Bottom
	Old Method	4:1	6.3±0.6	25.3±4.0	68.4±3.7
10	Control MPEG		5.5±0.3	9.1±4.9	85.4±4.8
	New Method	2:1	71.6±1.2	11.1±1.2	17.3±0.5
	Control MPEG		4.9±0.2	1.6±1.6	104.3±7.3
15					

The increase in interface partitioning with associated decrease in bottom phase partitioning with the old method indicated some PEGylation. However with the activated PEG made by the new method, even at the lower molar ratio there is extensive partitioning to the top phase indicating the highest degree of PEGylation of the two samples.

To increase the PEGylation with the rather unreactive old method sample, the molar ratio was increased to 20:1, using a final concentration of 35% w/w TMPEG. This caused the vesicles to aggregate. Controls with MPEG also showed aggregation. Addition of 75µl of the reaction mixture to 2.2 ml of N-tris-(hydroxymethyl)methyl-2-amino-ethane-sulphonic acid (TES) buffer [Fisher et al (1991) Cell and Model Membrane Interactions p.47 Plenum Press, New York] pH 7.4 gave solutions with OD₆₅₀ of 0.26 whereas untreated

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vesicles in control buffer had OD₆₅₀ of 0.01, indicating that the aggregation observed with the TMPEG and MPEG was not completely reversible. It is well established that PEG solutions can produce fusion and/or permeability increases
5 [Tilcock & Fisher (1982) Biochimica et Biophysica Acta, 688 645-652, Aldwinkle et al., (1992) ibid, 689: 548]

Example 9

(a) 1 μ g of the supercoiled plasmid pBR322 was
10 digested with 100 units of the restriction enzyme Rsa I for two hours at 37°C, in low salt buffer consisting of 10mM Tris HCl pH 7.5, 10mM MgCl₂, 1 mM dithiothreitol (DTT). This yields four blunt ended fragments per molecule of pBR322. Protein was removed by phenol/chloroform
15 extraction. After ethanol precipitation the DNA was resuspended in a small volume of tris-EDTA (TE) pH 7.6 and the DNA was end labelled with [γ 32P] dATP using a BCL kit as per the manufacturer's instructions. Removal of the free [32 P] dATP was achieved using Sephadex G-50 columns. The DNA
20 was then split into 6 aliquots. Three of these aliquots were made single stranded by boiling and then exposed to either TMPEG or MPEG, at a concentration of 400mg/ml in coupling buffer (consisting of 0.005M sodium phosphate 0.125M sodium chloride pH 7.5) or to coupling buffer only,
25 at a volume ratio of 150 μ l:150 μ l), for 20 minutes. Three other aliquots, which were allowed to remain double stranded, were incubated with either TMPEG, MPEG or

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coupling buffer only, under identical conditions. After incubation, all the samples were again made single stranded by boiling. The samples were examined in a 2% alkaline agarose gel, (50mM NaOH, 1mM EDTA pH 8.0) using alkaline loading buffer. This was a 6x stock consisting of 0.3M NaOH, 6mM EDTA pH 8.0, 18% Ficoll 400, and 0.15% bromocresol green. The samples were run overnight at 50v. The dried gel (Fig. 5) was analysed by autoradiography, the film was developed in an automated Fuji X-ray film developer. Lanes 1-6 from left to right are: 1-3 DNA exposed while double stranded; 4-6 DNA exposed while single stranded; 1&4 coupling buffer; 2&5 MPEG; 3&6 TMPEG. Only single stranded DNA exposed to TMPEG showed a mobility shift demonstrating coupling of PEG to the DNA.

15

(b) Eco RI digested pBR322 containing a single cleavage site yielding one double stranded fragment with a 3' recessed 4bp cut and the following single stranded overhangs:-

20

5'NC.....AATTCN3'

3'NGTTAA.....GN5'.

Of three aliquots, one was treated with a reaction mixture consisting of TMPEG (400 mg/ml) in coupling buffer (50 ml), DNA (circa 0.1 µg in 50 ul of tris EDTA). The coupling buffer (pH 7.5) was a solution of sodium chloride (0.125M) and sodium phosphate (0.05M). The reaction was conducted at

25

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room temperature for 20 min. A second aliquot was similarly treated with TMPEG (40 mg/ml) in coupling buffer and the third was treated with coupling buffer alone.

- 5 (c) Bam HI digested pBR322 containing a single cleavage site yielding one double stranded fragment with a 3' recessed 4bp cut and the following single stranded overhangs:-

5'NG.....GATCCN3'

10 3'NCCTAG.....GN5'.

This material was divided into three aliquots which were treated with two concentrations of TMPEG and coupling buffer as in (b).

- 15 To detect the extent of PEG-modification the staggered ended DNA was end-labelled with ³²P and then partitioned in a PEG-phosphate phase system to determine the partition coefficient (K), results being shown in Table 3.

20

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Table 3

	No PEG	TMPEG 40mg/ml	TMPEG 400mg/ml
5	DNA digest	EcoR1 50µg/ml	EcoR1 50µg/ml
	PEG-phase (%) *	41.7	58.4
	PO ₄ -phase (%) *	58.3	41.5
10	log(K)	-0.145	-0.148
	DNA digest	BamH1 50µg/ml	BamH1 50µg/ml
15	PEG-phase (%) *	41.7	51.0
	PO ₄ -phase (%) *	58.3	49.0
	log(K)	-0.145	-0.017
			0.356

20 * Yields corrected for differential recovery from the phases via recovery controls.

Up to saturation there is known to be a linear relationship between the number of PEG molecules attached per target
 25 molecule and log(K). The DNA cleaved with EcoRI (with two free amino groups supplied by two adenine bases) is more readily modified than the DNA cleaved with BamHI (with three free amino groups supplied by adenine, guanine and cytosine).

30

Example 10

BALB/c, male, 6-8 week-old mice, weighing approximately 20g, were pooled into groups of three per condition, with adlib access to food and water. PEG-modified GM-CSF samples

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were prepared as in Example 1, using the concentrations of GM-CSF and TM-PEG indicated in Table 4a. The reaction products were evaluated by FPLC and the proportions of individual species were estimated by fitting summated Gaussian curves to the FPLC profiles and estimating the area under the curve corresponding to each individual peak and expressing this as a % of the total area for the whole FPLC profile (Table 4b). The inventors have previously established that the individual peaks seen at low TMPEG concentrations (peaks 0, 1, 2 and 3) correspond to GM-CSF, PEG₁-GM-CSF, PEG₂-GM-CSF, PEG₃-GM-CSF, respectively. At high TMPEG concentrations the peaks are broader and less well resolved thus fitting of individual Gaussian curves over all regions of the FPLC profile may not be possible. Thus peak 3 and the peaks for more highly substituted GM-CSF (potentially PEG₄₋₇-GM-CSF) have been pooled. The peak eluting with the void volume peak is well resolved from the PEG₄₋₇-GM-CSF and presumably represents aggregates, the area of this peak was used to estimate the proportion of aggregated material (Table 4b).

The different PEGylated samples and unmodified GM-CSF (Hoechst), all containing <4% [¹²⁵I]GM-CSF (Amersham, 1222 Ci/mmol) to serve as a tracer, were administered at a dose of 0.0524μg in 100μl (3.45 x 10⁻¹² mole; equivalent to 2.5μg/kg body weight) by subcutaneous injection. The amount of TMPEG concomitantly administered is also indicated in Table 4a (MPEG has been shown not to influence t_{1/2}, but

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does influence egress from the subcutaneous site). Up to eight 25 μ l tail vein blood samples were collected from each mouse using capillary tubes at various time points ranging between 10 minutes and 80 hours, to give a minimum of three
5 samples per time point. Samples from the capillary tubes were washed immediately into 1ml of PBS containing 50 I.U. of heparin. Radioactivity was determined using a gamma counter (LKB 1270 Rackgamma) results being shown in Fig. 6a (open circles for untreated material, other symbols as
10 indicated on the figure). Clearances for the materials of reactions A and B were clearly biphasic, presumably reflecting the large proportion of unmodified material remaining in these preparations (Table 4b). Half-lives were calculated using linear regression (log concentration
15 versus time) for the slower clearing component if biphasic elimination was present (solid lines Figure 6a; dotted lines give the 95% confidence intervals). The $t_{1/2}$ values \pm SE are given in Table 5.

With the exception of the product of reaction D,
20 which is known to contain >50% of aggregates (the latter are known to clear slowly), $t_{1/2}$ for the other reactions (A-C) are similar. Although there may be some increment in $t_{1/2}$ between reactions A and B, surprisingly, there is no additional increment in $t_{1/2}$ between reactions B and C. This
25 was not anticipated in view of the fact that reaction C produced a higher proportion of PEG₂-GM-CSF and adducts with higher PEGylation ratios. The difference in $t_{1/2}$

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between reactions A and B is also not marked and, with the proviso that dissection of early and late components introduces more error into the $t_{1/2}$ estimates, it can be inferred that the addition of the first PEG molecule has the greatest impact on the clearance of GM-CSF. This is unexpected because the estimates for apparent molecular weight from FPLC were initially: GM-CSF 14.5kDa; PEG₁-GM-CSF 40.5kDa; PEG₂-GM-CSF 67.6 kDa; PEG₃-GM-CSF 112.9 kDa and after 3 or 4 independent estimates the values were: GM-CSF 12.5 \pm 1 kDa; PEG₁-GM-CSF 41.5 \pm 3.6 kDa; PEG₂-GM-CSF 75.2 \pm 10.5 kDa; PEG₃-GM-CSF 138.5 \pm 27.3 kDa. Thus, PEG₁-GM-CSF should not exceed the renal molecular weight threshold for excretion and the major increment in the $t_{1/2}$ would have been expected to be between PEG₁- and PEG₂-GM-CSF not between GM-CSF and PEG₁-GM-CSF. This result is also surprising in that the increment in apparent molecular weight is unexpectedly large for the addition of the first MPEG 5000 molecule (12.5 to 41.1 kDa). Other proteins (e.g. recombinant erythropoietin) have shown a surprisingly small increment in apparent molecular weight with the addition of one MPEG 5000 molecule (eg circa 1.4 kDa increment).

In order to establish the contributions to extended $t_{1/2}$ we examined the tissue distribution of material prepared in an identical manner to reaction C, using an identical dose. At each of the times indicated, one animal was sacrificed and organs/tissue including kidney, spleen, liver, lung, heart and femur were recovered and

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radioactivity was measured as above. Figure 6b shows the results. The dotted and dashed lines indicate the blood levels of GM-CSF and PEG-GM-CSF respectively. The filled circles and open circles are the organ concentrations of PEG-GM-CSF and GM-CSF respectively. Comparison of organ:blood concentration ratios indicates that renal accumulation (the kidney is the only organ which achieves high organ:blood ratios and is the site which is known to catabolise the peptide to small fragments), is substantially reduced by PEGylation of the degree achieved in reaction C. This is somewhat surprising since circa 24% of the reaction C mixture represents PEG₁-GM-CSF (i.e. material below the renal threshold). It suggests that the size of PEG conjugates is not the only factor in reduction of renal clearance. Thus the degree of substitution desirable for individual targets will have to be assessed on a case-by-case basis.

20

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Table 4(A)

Coupling Ratios and Compositions for PEGylated GM-CSF Preparations

5	PREPARATION	GM-CSF ($\mu\text{g/ml}$)	TMPEG (mg/ml)	GM-CSF (dose, μg)	TMPEG (dose, mg)
	Unmodified Control	0.524	0	0.0524	0
	Reaction A	0.524	6.46	0.0524	0.646
	Reaction B	0.524	19.75	0.0524	1.975
10	Reaction C	0.524	197.5	0.0524	19.75
	Reaction D	0.524	459.7	0.0524	45.97

15 Table 4(B)

Percentage of GM-CSF in different fractions at various coupling reactions.

20

25

	Unmodified GM-CSF (Peak 0)	PEG-modified-GM-CSF			
		Peak-1	Peak-2	Peak-3 & higher	Putative Aggregates
	Unmodified Control	100	0.0	0.0	0.0
	Reaction A	48.6	27.7	14.1	3.1
	Reaction B	31.0	26.0	21.6	8.0
30	Reaction C	16.8	23.5	15.9	19.2
	Reaction D	11.1	37.7*		
					51.2

* Combined data for all PEG-GM-CSF Peaks (incompletely resolved)

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Table 5

Half-life of late clearing components in PEGylated (GM-CSF)
5 preparations).

	$t_{1/2}$ (hours)	$t_{1/2} + SE^*$	$t_{1/2} - SE^*$
10 Unmodified Control	1.63	1.81	1.47
Reaction A	11.7 (7.2)	14.0 (8.6)	10.1 (6.2)
Reaction B	18.1 (11.1)	23.1 (14.2)	14.8 (9.1)
Reaction C	16.8 (10.3)	17.3 (10.6)	16.4 (10.1)
15 Reaction D	25.1 (15.3)	26.0 (15.9)	24.2 (14.8)

* calculated from the slope of the regression of log concentration versus time over portions of dose response curves (shown in fig.6(a)), \pm the standard error of the slope.

20 Figures in parentheses indicate the fold increase over the control figure.

Example 11.

25 A direct comparison using a 2h. coupling period and a molar ratio of activated PEG:lysine of 305:1 gave the following results (assessing modification by phase partitioning as described by Delgado (1990)) is shown in Table 6.

30

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Table 6

PEGYLATION METHOD	PARTITION COEFFICIENT
Unmodified Control	1.54±0.07
5 The present invention (TMPEG)	4.77±0.04
Cyanuric Chloride	9.0±0.74*
Carbonyldiimidazole	2.06±0.14
Phenylchloroformate	4.92±0.02
10 Succinimidyl succinate	3.35±0.25

* Result compounded by the presence of large amounts of cross-linked products for which log K will not be a linear function of modification.

15 These results demonstrate that a fast reaction rate is achieved with the present invention so that there is less time for a target material to be damaged by the possibly hostile environment of the coupling reaction.

20 Example 12

After storage at 4°C, ¹²⁵I-GM-CSF shows degradation with the appearance of label in low molecular weight material. This process is slowed by PEG modification.

25 Fig.7 shows FPLC profiles of representative examples stored for 1 day (upper panels) and 33 days (lower panels). When supplied, ¹²⁵I-GM-CSF has little or no low molecular weight labelled species. After storage for 1 day of an unmodified (open circles) preparation and a modified preparation
30 (exposed to TMPEG at a molar ratio of 305:1 as described

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above, filled circles) there was a small amount of labelled low molecular weight material (peak 3) in both preparations. In both, the majority of the material (peak 1) is high molecular weight (the size distribution difference is commensurate with the PEGylation of the intact protein and the low molecular weight material probably represents free iodine). After storage the low molecular weight peak had increased substantially and a second peak (peak 2) had appeared between it and the peak of unmodified material (lower panel). There has been generation of intermediate and low molecular weight fragments in both samples. The former are commensurate in size with proteolytic cleavage products and the latter either with free iodine or very small fragments (i.e. < circa 1kDa). The relative amounts of ^{125}I in the samples is listed in the Table 7. Pegylation reduces the loss of high molecular weight species.

Table 7

	Peak	Before Storage	After 1 Month Storage
GM-CSF	1	76.1	9.2
	2	0	20.6
	3	21.5	68.2
PEG-CM-CSF	1	81.1	45.8
	2	0	8.9
	3	12.4	36.3

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Example 13

Dry stored TMPEG, prepared as in Example 1 above, derived from an MPEG preparation (Union Carbide UK) contaminated with circa 22% PEG (when estimated by NMR) was used in this Example. This contaminating PEG results (during the polymer activation process) in the formation of biactivated tresyl PEG which produces aggregates in the final product; the formation of aggregates can be reduced by reducing the level of contamination of the MPEG preparation by PEG. The erythropoietin (EPO) preparation used was a commercial preparation obtained from Cilag Ltd and the erythropoietin stock contained 3200 u/ml in PBS. This preparation contains 3200 units of EPO, and 2 mg/ml of human serum albumin. The coupling ratios were calculated on the basis of the total number of available lysine groups in the erythropoietin and carrier protein, and would need to be adjusted accordingly for pure erythropoietin preparations. The erythropoietin preparation was exposed to the stated molar ratios of TMPEG:lysine and was incubated at room temperature for two hours on a rotary mixer. The sham-reacted control was similarly exposed to diluent, (PBS). The resulting samples were analysed by FPLC the profiles being shown in Fig.8 panels A to F; the vertical axis in all cases is C.P.M. [¹²⁵I]-erythropoietin:

A) Sham treated; B to F TMPEG:lysine at ratios B) 9.375:1; C) 18.75:1; D) 37.5:1; E) 75:1; F) 305:1

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Samples (200 μ l containing circa 10.5 μ g/ml total proteins) were analysed on a Pharmacia FPLC system with a Superose 12 HR 10/30 column previously equilibrated with PBS. The samples were loaded onto the column and then
5 eluted with sterile PBS at a flow rate of 0.3ml per minute; 0.25ml fractions were collected. To monitor protein profiles, PEG-EPO was prepared using [125 I]-erythropoietin (Amersham) and fractions were analysed using a gamma counter (Nuclear Enterprises NE1600). To check the
10 biological activity of a series of preparations made using the same TMPEG preparation, over a similar range of molar ratios of TMPEG:lysine, using unlabelled EPO (Cilag), serial dilutions of the unfractionated reaction mixture were exposed to the haemopoietic cell-line, TF-1, to induce
15 thymidine uptake. Cultures were performed in microtitre plates containing 50 μ l of cell suspension (5×10^4 cells/well) in RPMI-1640 medium containing 5% foetal calf serum (FCS) in round bottom microwell plates (NUNC). To increase sensitivity of TF-1 cells to cytokine, they were
20 deprived of their maintenance growth stimulus (GM-CSF at 50 units/ml) for 24 hours prior to assay. Each well contained either PEG-erythropoietin or unmodified erythropoietin, diluted in RMPI-1640. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide in air at 37°C
25 for 18 h. Cells were then exposed to 0.5 μ Ci of [methyl- 3 H]thymidine 25Ci/mmol. Amersham) in 50 μ l of culture medium and incubation was continued as above for 4 h. Cells were

- 82 -

harvested onto glass filters (Titertek Flow labs) using a Dynateck multimesh 2000 harvester, washed with methanol and dried for 12 h at room temperature. [³H]-thymidine incorporation was determined by scintillation counting

5 (Beckman LS 5000 CE) using 5ml Filter Count (Packard) the results being expressed as D.P.M. of the test sample minus D.P.M of unstimulated cells; negative values therefore indicate inhibition of background thymidine uptake.

Fig.9a shows the induction of thymidine uptake by all

10 preparations, both PEG-modified and unmodified. Fig. 9b shows the early portion of the dose-response curves for concentrations less than one unit/ml at expanded scale to facilitate comparison. All preparations produced stimulation of incorporation of thymidine, but there is a

15 progressive contamination by inhibitory material, whose activity is most evident at 3 and 10 units/ml of erythropoietin. The inhibitor is related to the TMPEG:lysine molar ratio suggesting that either TMPEG itself or co-product is responsible (TMPEG does have an

20 inhibitory effect in this assay system [Malik (1992)].. This inhibitory material was not evident to a similar degree in PEG-GM-CSF preparations, even those prepared with the same batch of TMPEG, and its nature has not yet been determined but may reflect altered sensitivity of the cell line to

25 inhibition under different growth conditions. The similarity of the upward portion of the dose-response curves indicates that there is little inherent loss of

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biological activity for all the preparations studied.

Example 14

5 NMR analysis showed that a standard preparation of MPEG-5000 (kindly donated by Union Carbide) contained 23.5 mole % PEG and 76.5 mole % MPEG. Method: ^1H -nmr spectra were acquired on a Bruker WN 259 spectrometer on polymer samples in DMSO-d_6 (50mg/ml). Integrations of the peaks at
10 4.56ppm (OH), 3.56 ppm ($\text{CH}_2\text{CH}_2\text{O}$) and at 3.26 ppm (OCH_3) were made and used to calculate the PEG and MPEG contents (also the molecular mass of the sample which was 5200Da).

PEG contamination results from its production as a by-product during the polymerisation reaction for the
15 manufacture of MPEG (hydroxide used to catalyse the reaction, initiates a side reaction yielding PEG). Since PEG has two termini at which polymerisation can take place, it should achieve a larger molecular weight than that of the MPEG. These data confirm that this side reaction takes
20 place largely during the early phase of the reaction, since the size distribution of the PEG product is approximately double that of the MPEG product and there is no substantial proportion of material spanning a range of intermediate sizes (as would be anticipated if the side reaction
25 continued throughout the polymerisation).

To confirm that the contaminating PEG is, as anticipated, responsible for aggregate formation during the

- 84 -

coupling reaction, a TMPEG preparation derived from the same MPEG preparation analysed above was fractionated on the expectation that it would contain circa 25.5% bi-activated PEG. [Such fractionation requires prolonged exposure to an aqueous environment and resulted in some overall loss of activity (thus requiring appropriate sham-treatment of unfractionated controls) and is therefore not advisable as a step in the process of the invention, rather purification prior to activation is recommended]. The TMPEG was then exposed to ^{125}I -gm-CSF under standard coupling conditions as described over a range of molar ratios as indicated in Table 8 (aggregate formation is more likely at higher molar ratios and/or concentrations of polymer and target). Aggregates were identified by FPLC on Superose 12 (as labelled material eluting with the void volume) and PEG-modified GM-CSF species (as labelled material eluting faster than unmodified GM-CSF, but not as fast as the aggregated material). This identification has been confirmed independently by phase partitioning. The fractionation by vesicle chromatography separates material less prone to produce aggregates (permeable fraction) and more prone (excluded fraction) than the starting material respectively. This validates two points; first that the PEG becomes biactivated and second that the reduction of the contaminating PEG by size fractionation will reduce the amount of aggregates. Since the acceptable level of aggregates will vary from application to application a

- 85 -

limit for contamination of MPEG by PEG will have to be selected on a case-by-case basis.

Table 8

5

Reduction of PEG contamination reduces aggregate formation. Aggregates are expressed as % total PEG-modified material (aggregates + PEG_n-gm-CSF where n=1-7)

10

Table 8

	Unfractionated MPEG	Permeable Fraction	Excluded Fraction
	PREP 1 (150:1 TMPEG:lysine)		
15	Aggregates	21.6	6.1
	PREP 2 (120:1) TMPEG:lysine)		
	Aggregates	17.9	0
20	PREP 3 (17:1 TMPEG:lysine)		
	Aggregates	0	13.3

N.T. = Not Tested

25

30

35

40

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Claims

1. A process for producing an adduct of a polymer and a target material which process comprises the steps of

5

(a) reacting either

(i) an activating compound of formula (I)

10

X-AM (I)

wherein

15

-AM is an activating sulphonyl ester moiety optionally bearing a group for covalent bonding to a solid support, the solvolysis substituent constant of the group -AM being less than that of the trifluoromethane sulphonate group, and

X is a leaving group

20 or

(ii) reacting a solid support bearing moieties of formula (I')

25

X-AM'- (I')

wherein

30

-AM'- is activating sulphonyl ester moiety covalently bound to the solid support, the group -AM'- being such that the solvolysis substituent constant of the group -AM'- is less than that of the trifluoromethane sulphonate group, and

X is as defined above

35

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with a polymer of formula (II)



5 wherein

POL is a polymer moiety of valency $c+g$,

C is a capping group and c is zero or a positive number and

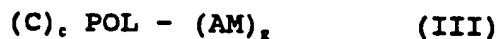
10

G is a terminal hydroxyl group reactive with the compound of formula (I) and g is a positive number

so as to form

15

(i) a sulphonate ester-activated polymer of formula (III)



20

wherein

C, POL, -AM, c and g are as defined above

25

and the group(s) -AM are linked to termini of the polymer as sulphonate esters of terminal hydroxyl groups

or

30

(ii) a solid support-bound, sulphonate ester-activated polymer of formula (III')



35

SS

- 88 -

wherein

C, POL, -AM'-, c and g are as defined above,

5 SS is a solid support and z is the number of
sulphonate ester-activated polymer moieties on the
solid support and

10 the groups -AM'- are linked to termini of the
polymer as sulphonate esters of terminal hydroxyl
groups,

and, when -AM bears a group for covalent bonding to a solid
support, reacting the sulphonate ester-activated polymer of
formula (III) with a solid support to form a solid support-
15 bound, sulphonate ester-activated polymer of formula (III')
as defined above

(b) reacting the sulphonate ester-activated polymer of
formula (III) or (III') with the target material and
20

(c) recovering the adduct of the polymer and the target
material,

in which process:

25

(i) the polymer of formula (II) is dry as
determined by benzene distillation,

30 (ii) the reaction of the compound of formula (I)
or (I') with the polymer of formula (II) is
conducted in an organic solvent which is
inert to the reagents and to the product of
formula (III) or (III') and is anhydrous as
obtainable using molecular sieves of 0.3nm;

35

(iii) the reaction of the compound of formula (I)

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or (I') with the polymer of formula (II) is conducted in a reaction vessel from which water is excluded;

- 5 (iv) the sulphonate ester-activated polymer of
 formula (III) or (III') so produced is
 recovered and either used directly in step
 (b) or stored, prior to use in step (b), in
10 the presence of a desiccating agent more
 hygroscopic than the product of formula (III)
 or (III'); and
- v) the reaction of the sulphonate ester-
 activated polymer with the target material is
15 conducted in a non-denaturing medium and non-
 denaturing temperature with respect to the
 target material.
- 20 2. A process according to claim 1 which is conducted in
 the liquid phase, using a compound of formula (I) in step
 (a).
3. A process according to claim 1 which is conducted at a
25 solid:liquid interface, using a solid support-bearing at
 least one moiety of formula (I') in step (a).
4. A process according to any one of claims 1 to 3
 comprising, in step (b), reacting, in predetermined molar
30 ratio and at predetermined concentrations, a sulphonate
 ester-activated polymer having two moieties -AM with a
 first target material (target¹), so as to produce a
 sulphonate ester-activated 1:1 adduct of polymer and
 target¹, then reacting the 1:1 adduct with a second target
35 material (target²) so as to produce a
 target¹:polymer:target² adduct, wherein the first and second

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target materials may be the same or different, and then recovering the adduct in accordance with step (c).

5. A process according to any one of claims 1 to 3
5 comprising reacting a sulphonate ester-activated polymer bearing a plurality of activating sulphonyl moieties -AM with a first target material, wherein the proportion of the activating sulphonyl moieties -AM on the sulphonate ester-activated polymer which react with the first target
10 material is controlled such that in at least a proportion of the molecules of intermediate polymer:target adduct produced there remain unreacted activating sulphonyl moieties -AM, and wherein such unreacted reactive groups -AM are then reacted with one or more different further
15 target materials so as to form materials polymer:[target]_n, wherein n is 2 or more and the first and further target entities are different; or the unreacted activating sulphonyl moieties are reacted with more of the first target material so as to form materials polymer:[target]_n,
20 wherein n is 2 or more and the target entities are all the same; or the unreacted activating sulphonyl moieties are reacted with further unreacted groups in the first target entity of the intermediate polymer:target adduct under conditions which hinder intermolecular reactions, such that
25 the polymer moieties are bonded at two or more positions to the target entity.

6. A process according to any one of claims 1 to 3
comprising, in step (b), reacting, in predetermined molar
30 ratio and at predetermined concentrations, a sulphonate ester-activated polymer having only one moiety -AM or -AM'- with a target material having more than one reactive group so as to form an adduct of the polymer and target having a preselected number of polymer molecules per molecule of
35 target, or having a preselected proportion of the reactive groups reacted with and linked to polymer molecules, and

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then recovering the adduct in accordance with step (c).

7. A process according to any one of claims 1 to 3 comprising, in step (b), reacting, in predetermined molar ratio and at predetermined concentrations, a sulphonate ester-activated polymer having two or more activating sulphonyl moieties -AM with a target material having two or more reactive groups so as to produce a sulphonate ester-activated 1:1 adduct of polymer and target then changing the reaction conditions so as to hinder intermolecular reactions whilst permitting intramolecular reactions between activating sulphonyl moieties -AM on the polymer and reactive groups on the target so as to form adducts of polymer and target having two or more covalent bonds between the polymer and target moieties, and then recovering the adduct in accordance with step (c).

8. A process according to any preceding claim wherein X is halogen.

20

9. A process according to any preceding claim wherein X is chlorine.

10. A process according to any preceding claim wherein the activating moiety -AM is selected from:

25 2,2,2-trifluoroethanesulphonyl,
pentafluorobenzenesulphonyl,
fluorosulphonyl,
2,4,5-trifluorobenzenesulphonyl,
30 2,4-difluorobenzenesulphonyl,
2-chloro-4-fluorobenzenesulphonyl,
3-chloro-4-fluorobenzenesulphonyl,
4-amino-3-chlorobenzenesulphonyl,
4-amino-3-fluorobenzenesulphonyl,
35 o-trifluoromethylbenzenesulphonyl,
m-trifluoromethylbenzenesulphonyl,

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p-trifluoromethylbenzenesulphonyl,
2-trifluoromethoxybenzenesulphonyl,
4-trifluoromethoxybenzenesulphonyl, and
5-fluoro-2-methylbenzenesulphonyl groups.

5

11. A process according to claim 10 wherein the group -AM is a 2,2,2 trifluoroethane-sulphonyl group.

12. A process according to any one of claims 1 to 9 wherein
10 the activating moiety -AM is a 2',3',5',6'-tetrafluoro-4-amino-phenyl-azobenzene-4'-sulphonic halide.

13. A process according to claim 12 wherein -AM is
2',3',5',6'-tetrafluoro-4-amino-phenyl-azobenzene-4'-
15 sulphonic chloride.

14. A process according to any preceding claim wherein the polymer is selected from polyalkylene compounds, polyvinyl compounds, polyacrylic compounds, polyionic compounds,
20 polyalkylene polyols, non-ionic surfactants, polyamino acids, synthetic polypeptides, polysaccharides, crosslinked products thereof and polysaccharide containing material; other organic polymers and inorganic polymers and block copolymers thereof.

25

15. A process according to claim 14 wherein the polymer is selected from poly(oxymethylene), polyethyleneglycols and oxides, methoxypolyethyleneglycols, polymethylethylene-glycol, polyhydroxypropyleneglycol, polypropyleneglycols
30 and oxides, polymethylpropyleneglycol, polyhydroxy-propyleneoxide, straight-chain and branched-chain polypropyleneglycols and derivatives thereof, polyethyleneglycol and polypropyleneglycol and the monomethyl ethers, monocetyl ethers, mono-n-butyl ethers,
35 mono-t-butylethers and monooleyl ethers thereof, esters of polyalkyleneglycols with carboxylic acids and dehydration

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condensation products of the polyalkyleneglycols with amines and other polyalkylene oxides and glycols and derivatives thereof, poly (vinylpyrrolidone), polyvinyl alcohol, poly (vinyl acetate), the copolymer poly (vinyl acetate-co-vinyl alcohol), polyvinylloxazolidone, poly (vinylmethyloxazolidone and poly (vinyl methyl ether), poly(acrylic acid)s, poly(methacrylic acid)s, polyhydroxyethylmethacrylates, Poly(acrylamide and poly(methacrylamide) and other amides thereof, poly(N,N-dimethylacrylamide), poly(N-isopropylacrylamide), poly(N-acetamidoacrylamide) and poly(N-acetamidomethacrylamide, and other N-substituted derivatives of the amides, poly(ethyleneimine), poly(ethylsulphonic acid), poly(silicic acid), poly(styrenesulphonic acid), poly(vinyl amine), poly(2-vinylpyridine) and its N-alkyl derivatives, poly (4-vinylpyridine) and its N-alkyl derivatives, poly(vinylsulphuric acid), poly(vinyl alcohol-co-vinyl sulphuric acid), poly(diallyldimethylammonium chloride), poly((dimethylimino) trimethylene(dimethylimino)hexamethylene dibromide), poly(ethylenephosphonic acid), poly(maleic acid), poly(2-methacryloyloxyethane-1-sulfonic acid), poly-(3-methacryloyloxypropane-1-sulfonic acid), poly(4-vinylbenzoic acid), poly(4-vinylbenzyl-trimethylammonium), poly[3-(vinyl-oxy)propane-1-sulphonic acid)], poly(4-vinylphenol)(poly[p-hydroxystyrene]), poly(4-vinylphenyl sulphuric acid), poly-(2-vinyl piperidine), poly(4-vinylpiperidine), poly(N-vinyl-succinamidic acid), polyoxyethylated glycerol, polyoxyethylated sorbitol, poly sorbates and polyoxyethylated glucose, polyoxyethylene-alkylphenols, polyoxyethylenemercaptans, polyoxyethylene- alkylamines, polyoxyethylene-alkylamides, polymers of D-glutamic acid and D-lysine, polylysine, polyalanine, polyglutamic acid, polyaspartic acid, polyproline, branched or unbranched polysaccharides comprising saccharide monomers selected from glucose, mannose, galactose, fucose,

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fructose, xylose, arabinose, glucuronic acid, sialic acid (neuraminic acid), galacturonic acid, mannuronic acid, D-glucosamine and galactosamine, dextran, dextran sulphate, p-aminoethyl cross-linked dextran, carboxymethyl dextran
5 and other dextran derivatives, cellulose, methyl cellulose, carboxymethyl cellulose and other cellulose derivatives, starches (amylopectin and amylose) and dextrans derived from starch, hydroxyethyl starches, agarose, ficoll and its carboxy methyl derivatives, glycosaminoglycan chains of
10 proteoglycans selected from hyaluronic acid, chondroitin sulphates, dermatan sulphate, heparin, heparin fragments, heparin oligosaccharides, heparan sulphate and keratan sulphate, carbohydrate-containing side chains of glycoproteins and glycolipids selected from gangliosides,
15 globosides and sulphatides, polysorbitol, polymannitol and other polymers of sugar alcohols, polysaccharides selected from glycogen, glucans, laminaran and glycosaminoglycans, polysaccharide sidechains of glycoproteins and glycolipids, alginic acid, polymannuronic acid, carrageenan, agar and
20 other sulphated polysaccharides, and other algal polysaccharides, pectins, plant gums, guaran and other seed mucilages, xanthans, gellan, alginate, scleroglucan, schizophyllan, curdlan, pullulan and other bacterial and fungal polysaccharides, polymers and copolymers of amines,
25 olefins, esters, acetal, polyamides, carbonates, ethers and phenylene sulphides, silicones, urea formaldehyde condensation products, phenol formaldehyde condensation products, urethanes, melamine formaldehydes, epoxy resins, acrylic resins, allyl resins, polyacrylic acid and
30 carbomers, silicates and other inorganic polymers containing organic moieties, block copolymers of polyethylene/ polypropylene-glycol, block copolymers of ethylene and maleic anhydride, block copolymers of polyalkylene glycols and polyvinylpyrrolidone or polyvinyl
35 alcohol, block copolymers of polyoxyethylene and polyoxypropylene, block copolymers of the ethers, esters or

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dehydration condensation products of polymers of ethylene glycol and propylene glycol, and block copolymers of acrylamide and acrylic acid.

- 5 16. A process according to any preceding claim wherein the target is selected from proteins, antibodies and fragments thereof, , IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and other interleukins and subtypes thereof, and other cytokines and derivatives or fragments thereof, granulocyte-macrophage colony stimulating factor, the alpha and beta forms of granulocyte-colony stimulating factor, macrophage-colony stimulating factor, and other colony stimulating factors, erythropoietin, haemopoietin-alpha and kit-ligand and other haemopoietins, IFNalpha, 15 IFNbeta and IFNgamma and other interferons, epidermal growth factor, platelet derived growth factor, transforming growth factor (alpha and beta forms), amphiregulin, somatomedin-C, bone growth factor, fibroblast growth factors, insulin-like growth factors, heparin binding 20 growth factors, tumour growth factors and other growth factors and bifunctional growth modulators, macrophage differentiating factor, differentiation inducing factor (DIF), leukaemia inhibitory factor and other differentiation factors, platelet activating factor, 25 macrophage activation factor, and other activating factors, heparin, proteases and their pro-factors, clotting factors VII, VIII, IX, X, XI and XII, antithrombin III, protein C, protein S, streptokinase, urokinase, prourokinase, tissue plasminogen activator, fibrinogen, hirudin, other 30 fibrinolytic/anticoagulant agents and other coagulation factors, peptide hormones, enzymes, vaccines, transcription factors and transcriptional modulators, carbohydrates, glycosoaminoglycans, glycoproteins and polysaccharides, phosphatidylethanolamine and phosphatidylserine and 35 derivatives thereof, sphingosine, cholesterol and other steroids and derivatives thereof, nucleotides, nucleosides,

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heterocyclic bases, DNA, RNA, synthetic and non-synthetic oligonucleotides, vitamins, antibiotics, bacteristatic and bactericidal, antifungal, anthelmintic agents, noradrenalin, alpha adrenergic receptor ligands, dopamine
5 receptor ligands, histamine receptor ligands, GABA/benzodiazepine receptor ligands, serotonin receptor ligands, leukotrienes and tri-iodothyronine and other small effector molecules, doxorubicin, methotrexate and other cytotoxic agents and derivatives thereof.

10

17. A process according to any one of the preceding claims comprising the steps of

15

(a) reacting dry MPEG (as determined by benzene distillation) containing less than 10% by weight of PEG (as determined by nmr and/or
chromatography) with tresyl chloride in dry dichloromethane (as determined using 0.3 nm
molecular sieves) in the presence of pyridine in a reaction vessel from which water is excluded,
20 so as to form TMPEG, recovering the TMPEG so formed and either storing it over phosphorus pentoxide or using it directly in step (b),

20

(b) reacting the TMPEG so formed, optionally after storage, with a target material such as erythropoietin (EPO) in aqueous medium and about 20°C and

25

(c) recovering the Target:MPEG adduct (eg EPO:MPEG) so formed.

30

18. A polymer:target adduct obtainable by the process of any one of the preceding claims, having polymer and target moieties directly linked by stable, non biodegradable
35 covalent bonds.

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19. A liposome bearing linear polymeric linking groups bound to the surface of the liposome at one terminal of the polymer and bearing a receptor ligand, receptor molecule, antibody, antigen or other molecule of therapeutic or
5 diagnostic interest at the other terminal.
20. A construct comprising a plurality of target molecules linked to a single polymer moiety, the polymer being a linear polymer having two reactive termini linked to two
10 target molecules or being a branched polymer having three or more reactive termini or a polymer having reactive pendant groups linked to three or more target molecules so as to enhance the biological activity thereof.
- 15 21. A construct according to claim 20 wherein the target molecules are different.
22. A construct comprising a polymer moiety linked to a target molecule at both termini of a linear polymer or to
20 two or more pendant reactive groups of a polymer or the termini of a branched polymer, at two, three or more sites on the target molecule.
23. A product for use in a therapeutic or diagnostic
25 method of treatment of the human or animal body.
24. Use of a product in the manufacture of a medicament for use in a therapeutic or diagnostic method of treatment of the human or animal body.
30
25. A pharmaceutical composition comprising a product according to any one of claims 18 to 22 or produced in accordance with the process of any one of claims 1 to 17 or obtainable by the process of any one of claims 1 to 17
35 together with pharmaceutically acceptable diluents or carriers.

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5

Abstract

10

Polymer modification

A process for producing adducts of a polymer and a target material which process comprises the steps of

15

(a) reacting either

(i) an activating compound of formula (I)



20

wherein

25

-AM is an activating sulphonyl ester moiety optionally bearing a group for covalent bonding to a solid support, the solvolysis substituent constant of the group -AM being less than that of the trifluoromethane sulphonate group, and

X is a leaving group

30

or (ii) reacting a solid support bearing moieties of formula (I')



35

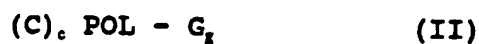
wherein

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5 -AM'- is an activating sulphonyl ester moiety covalently bound to the solid support, the group -AM'- being such that the solvolysis substituent constant of the group -AM'- is less than that of the trifluoro- methane sulphonate group, and

X is as defined above

10 with a polymer of formula (II)



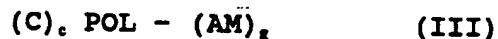
wherein

15 POL is a polymer moiety of valency $c+g$, C is a capping group and c is zero or a positive number and

20 G is a terminal hydroxyl group reactive with the compound of formula (I) and g is a positive number so as to form

(i) a sulphonate ester-activated polymer of formula (III)

25

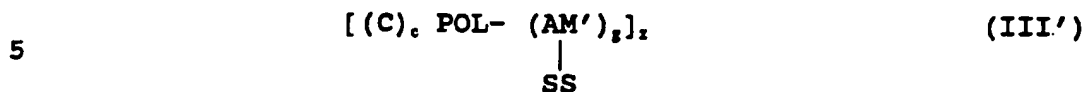


wherein

30 C, POL, -AM, c and g are as defined above and in which the group(s) -AM are linked to termini of the polymer as sulphonate esters of terminal hydroxyl groups

35 or (ii) a solid support-bound, sulphonate ester-activated polymer of formula (III')

- 100 -



wherein

10 C, POL, -AM'-, c and g are as defined above,
 SS is a solid support and z is the number of
 sulphonate ester-activated polymer moieties on the
 solid support

15 and the groups -AM'- are linked to termini of the
 polymer as sulphonate esters of terminal hydroxyl
 groups,

and, when -AM bears a group for covalent bonding to a solid
 20 support, reacting the sulphonate ester-activated polymer of
 formula (III) with a solid support to form a solid support-
 bound, sulphonate ester-activated polymer of formula (III')
 as defined above

25 (b) reacting the sulphonate ester-activated polymer of
 formula (III) or (III') with the target material and

(c) recovering the adduct of the polymer and the target
 material,

30

in which process:

(i) the polymer of formula (II) is dry as
 determined by benzene distillation,

35

(ii) the reaction of the compound of

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formula (I) or (I') with the polymer of
formula (II) is conducted in an organic
solvent which is inert to the reagents and to
the product of formula (III) or (III') and is
anhydrous as obtainable using molecular
sieves of 0.3nm;

(iii) the reaction of the compound of formula
(I) or (I') with the polymer of formula
(II) is conducted in a reaction vessel
from which water is excluded;

(iv) the sulphonate ester-activated
polymer of formula (III) or (III') so
produced is recovered and either used
directly in step (b) or stored, prior to
use in step (b), in the presence of a
desiccating agent more hygroscopic than
the product of formula (III) or (III');
and

(v) the reaction of the sulphonate ester-
activated polymer with the target
material is conducted in a non-
denaturing medium and non-denaturing
temperature with respect to the target
material.

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Fig.1a.

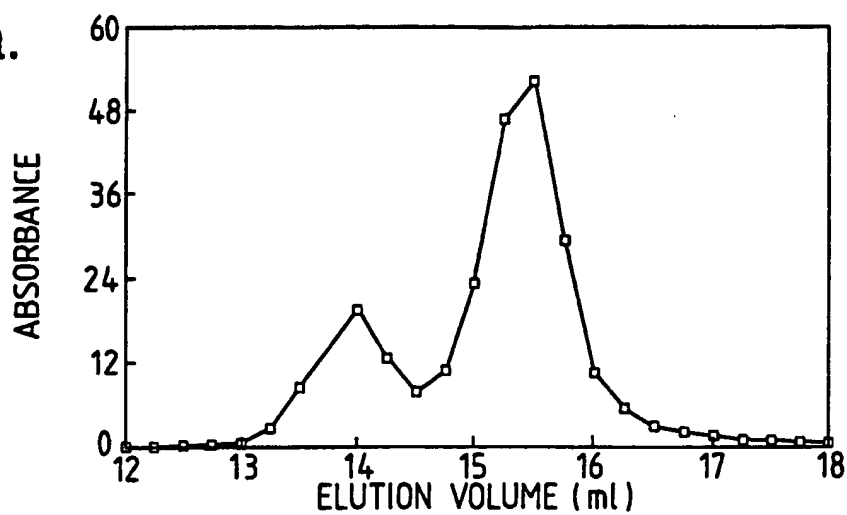


Fig.1b.

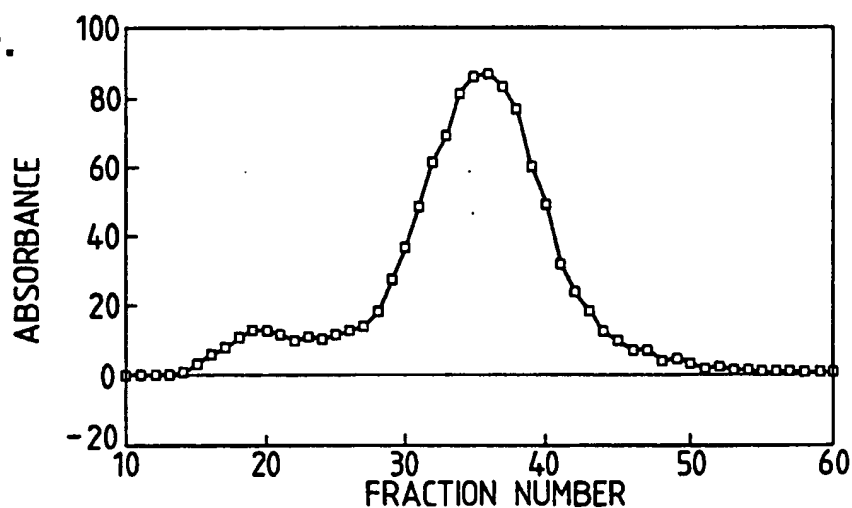
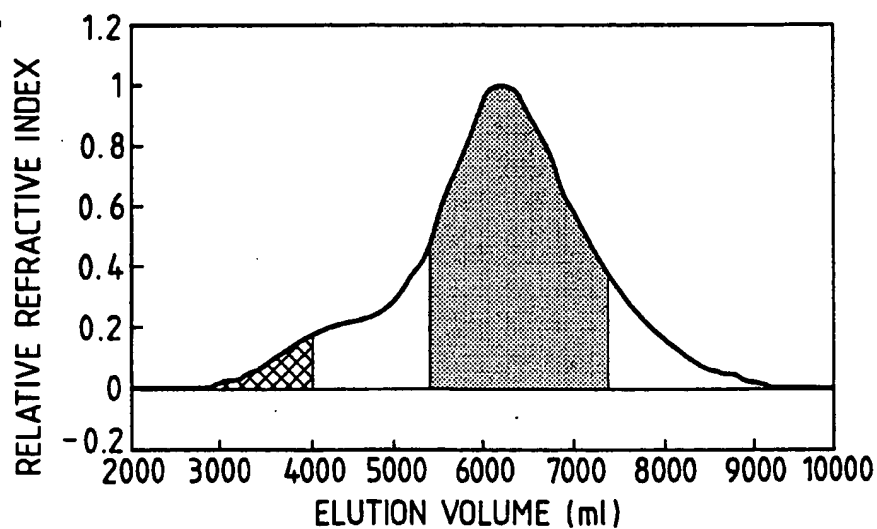


Fig.1c.



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Fig.1d.

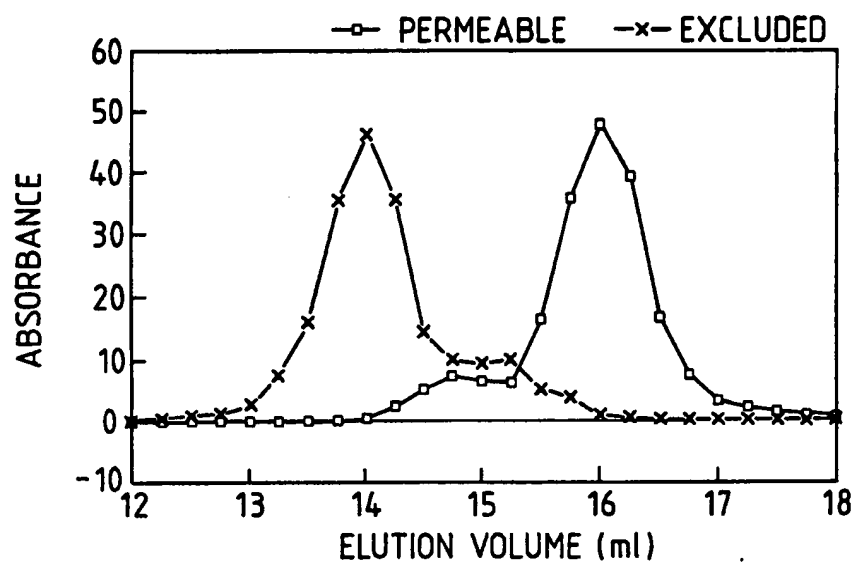
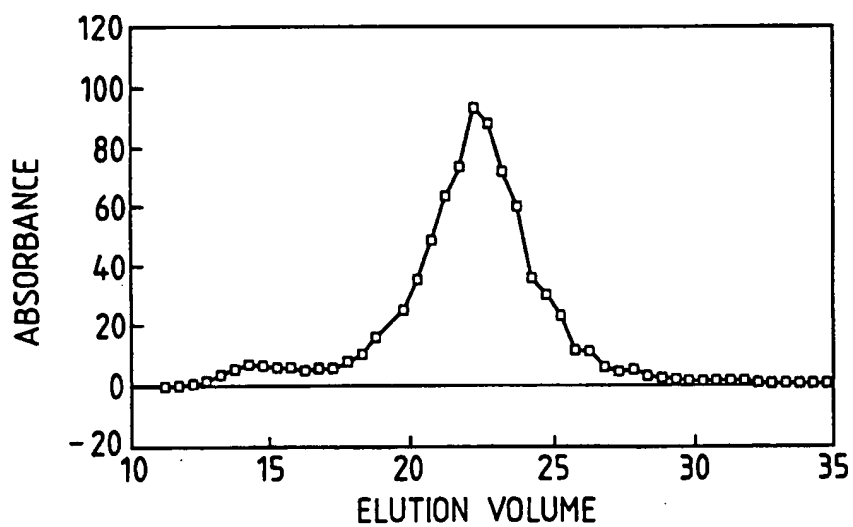
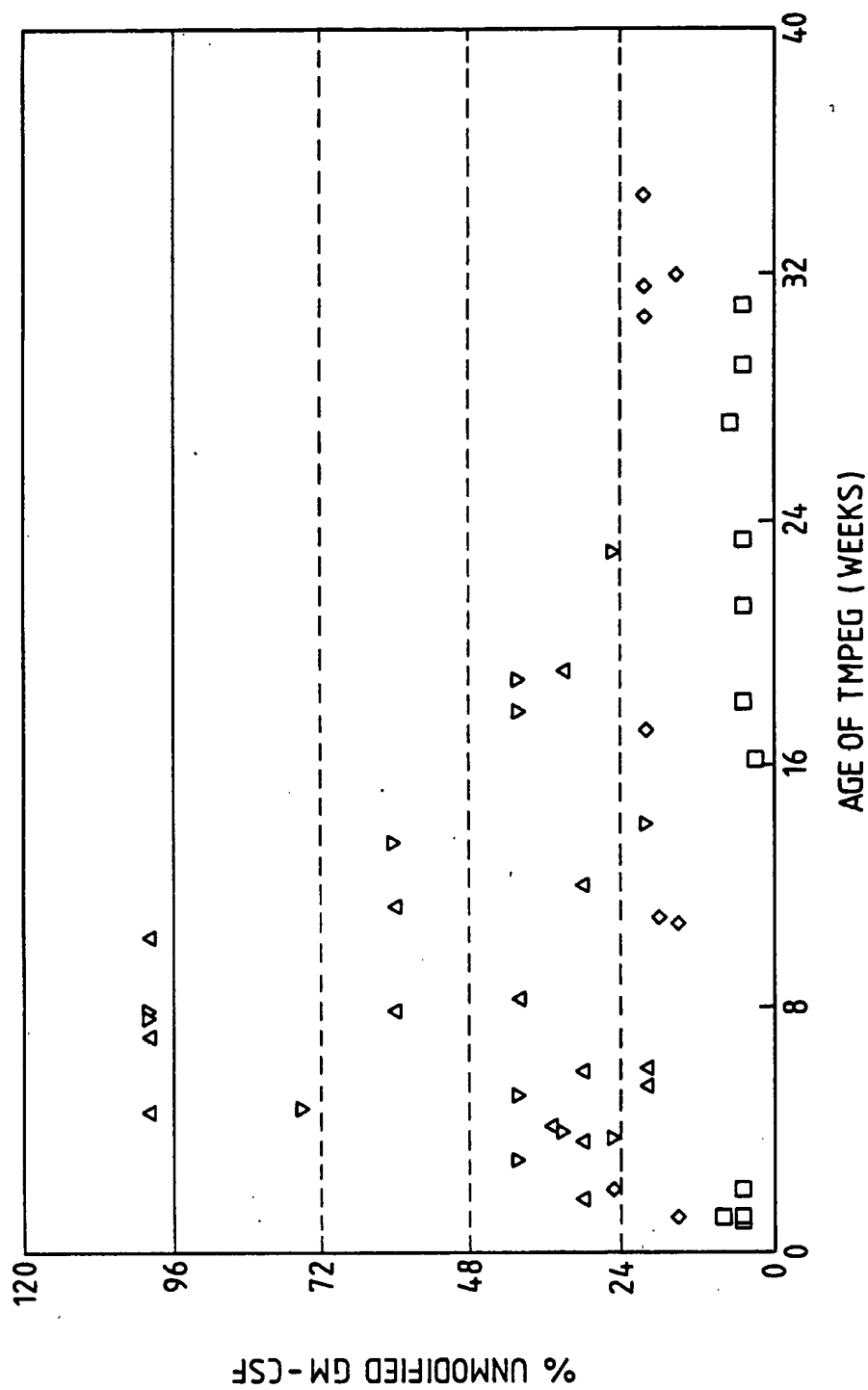


Fig.1e.



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Fig.2.



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Fig.3a.

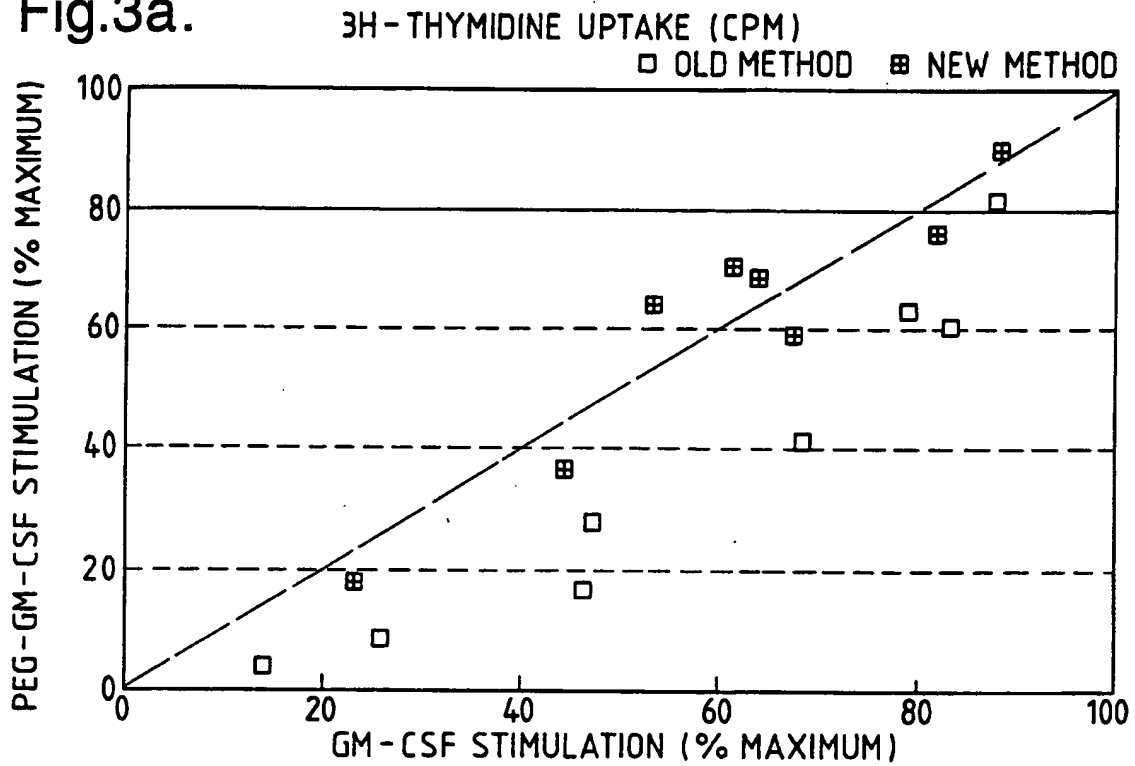
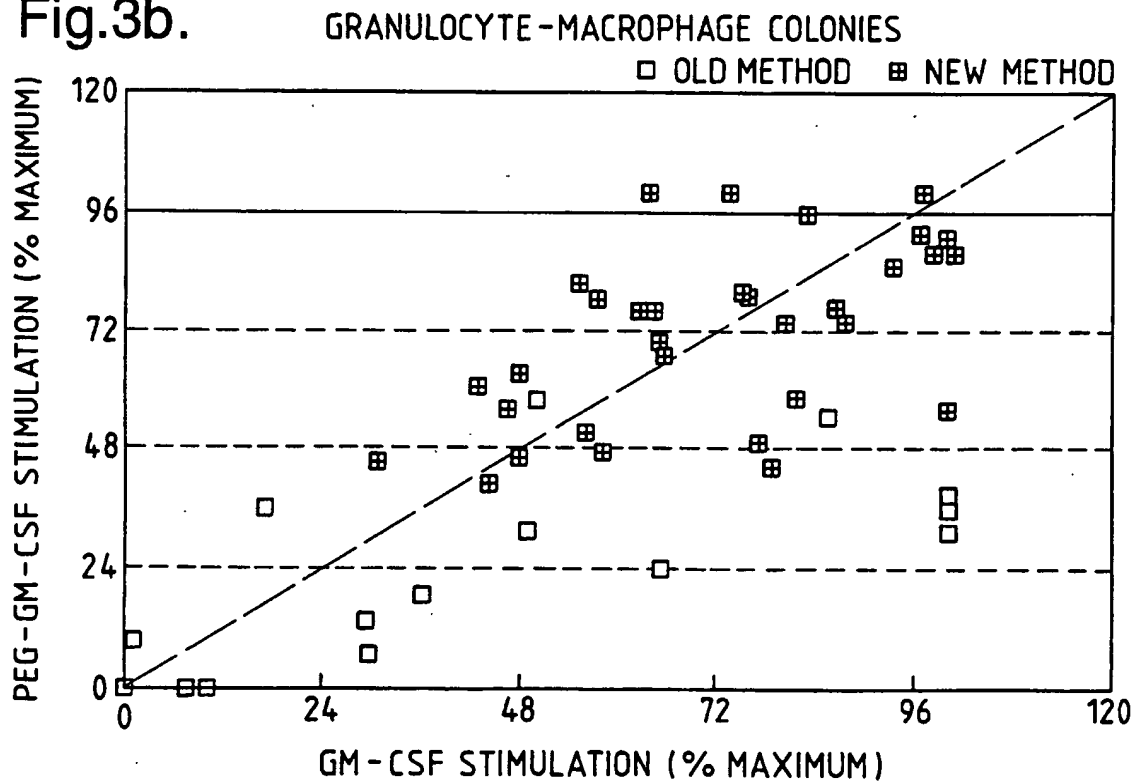
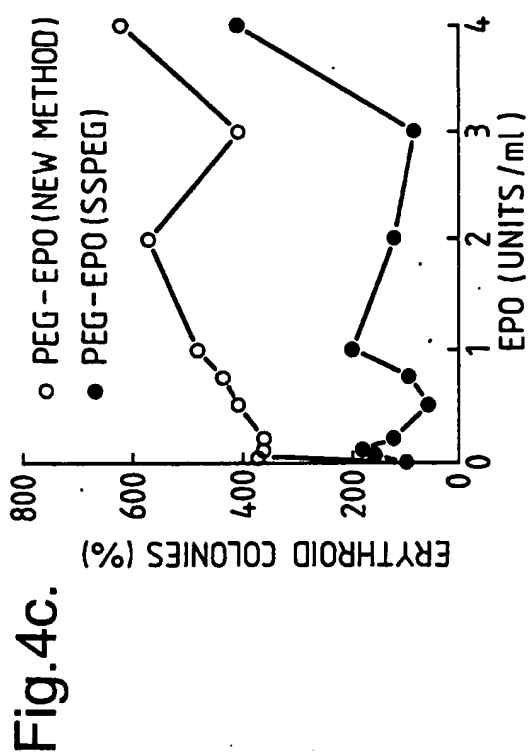
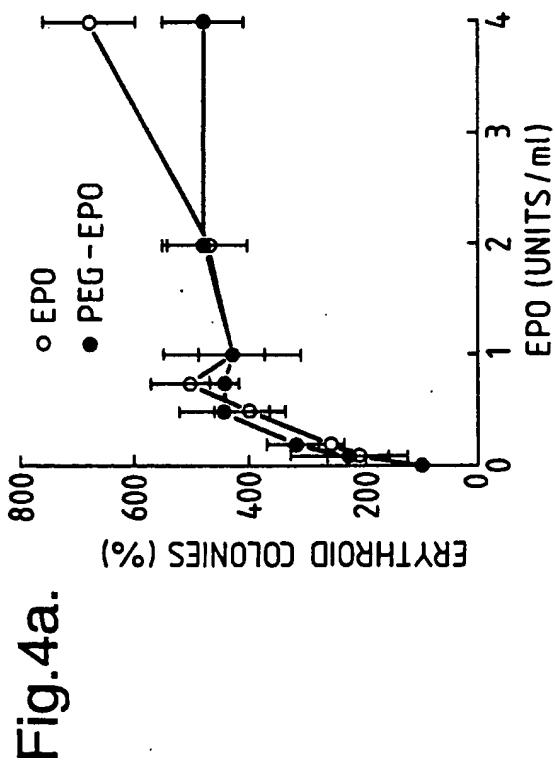
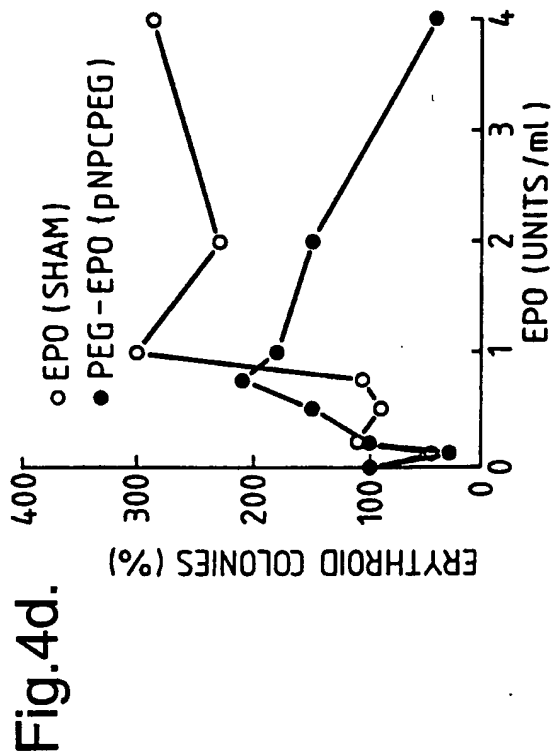
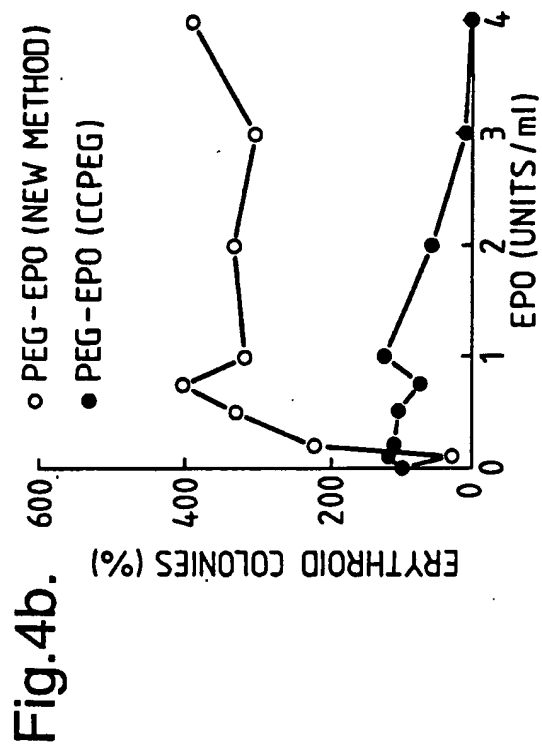


Fig.3b.



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Fig.5.

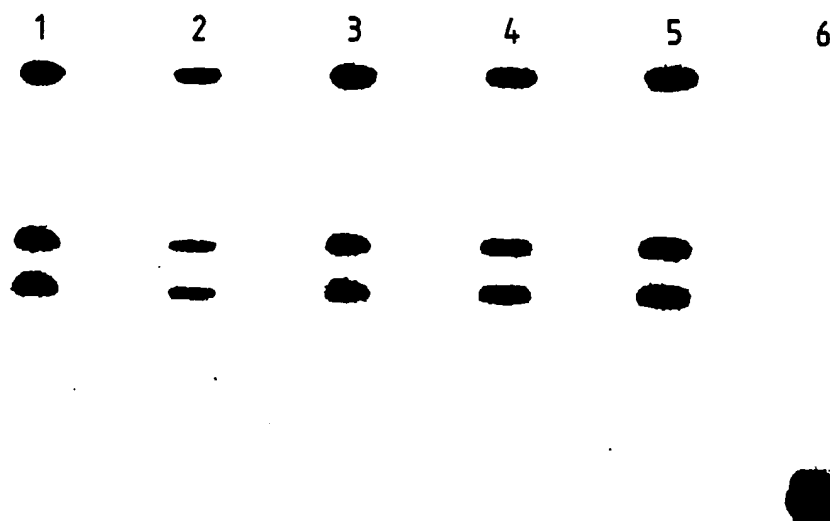
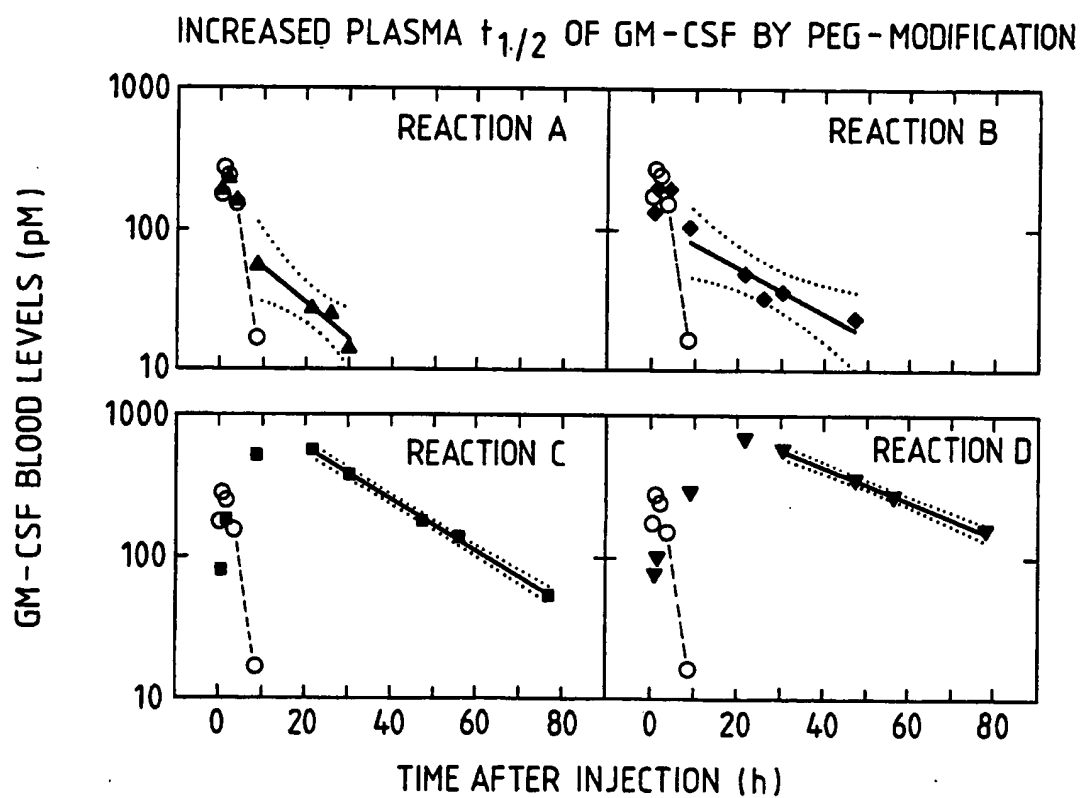


Fig.6a.



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Fig.6b.

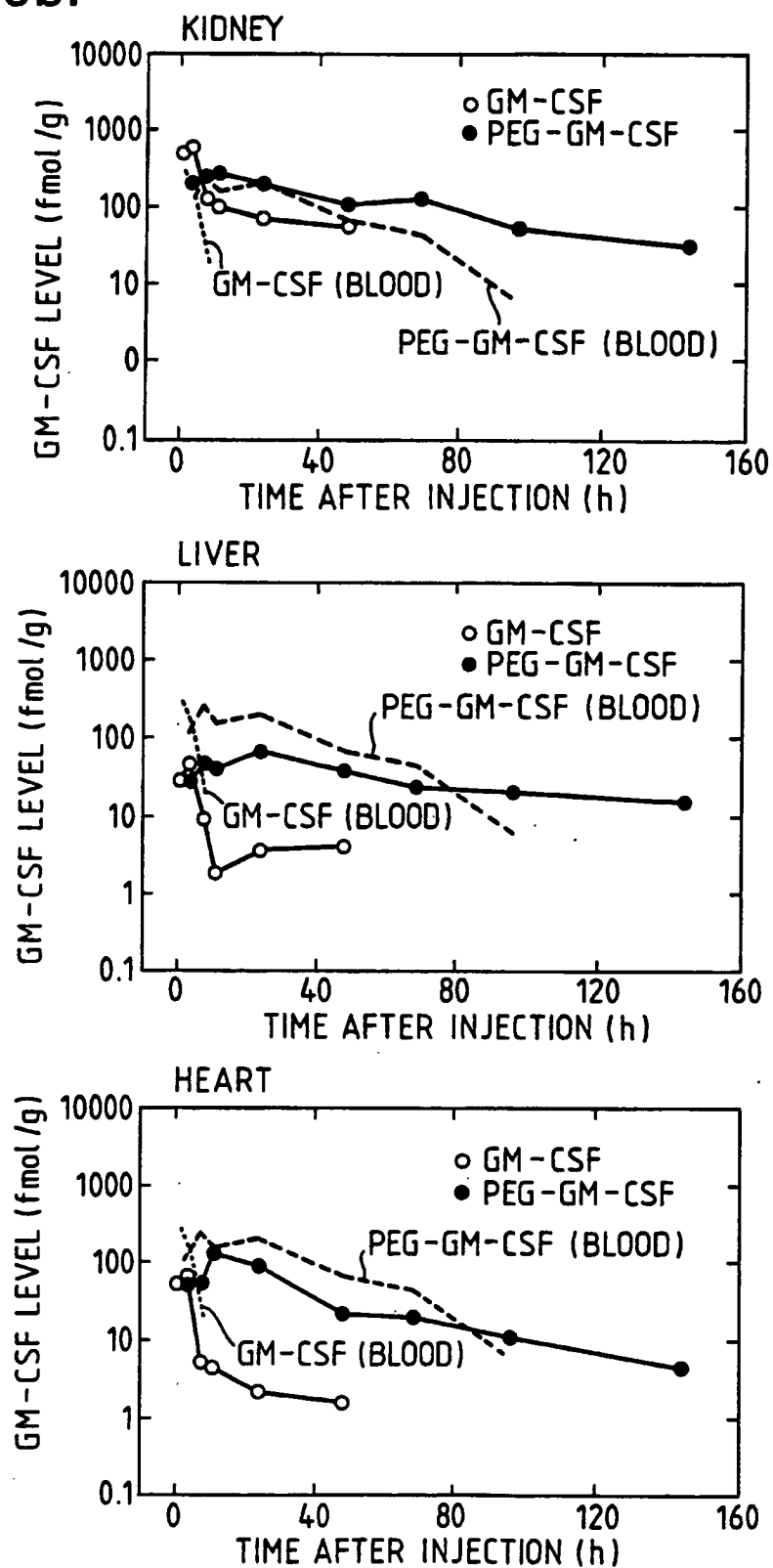
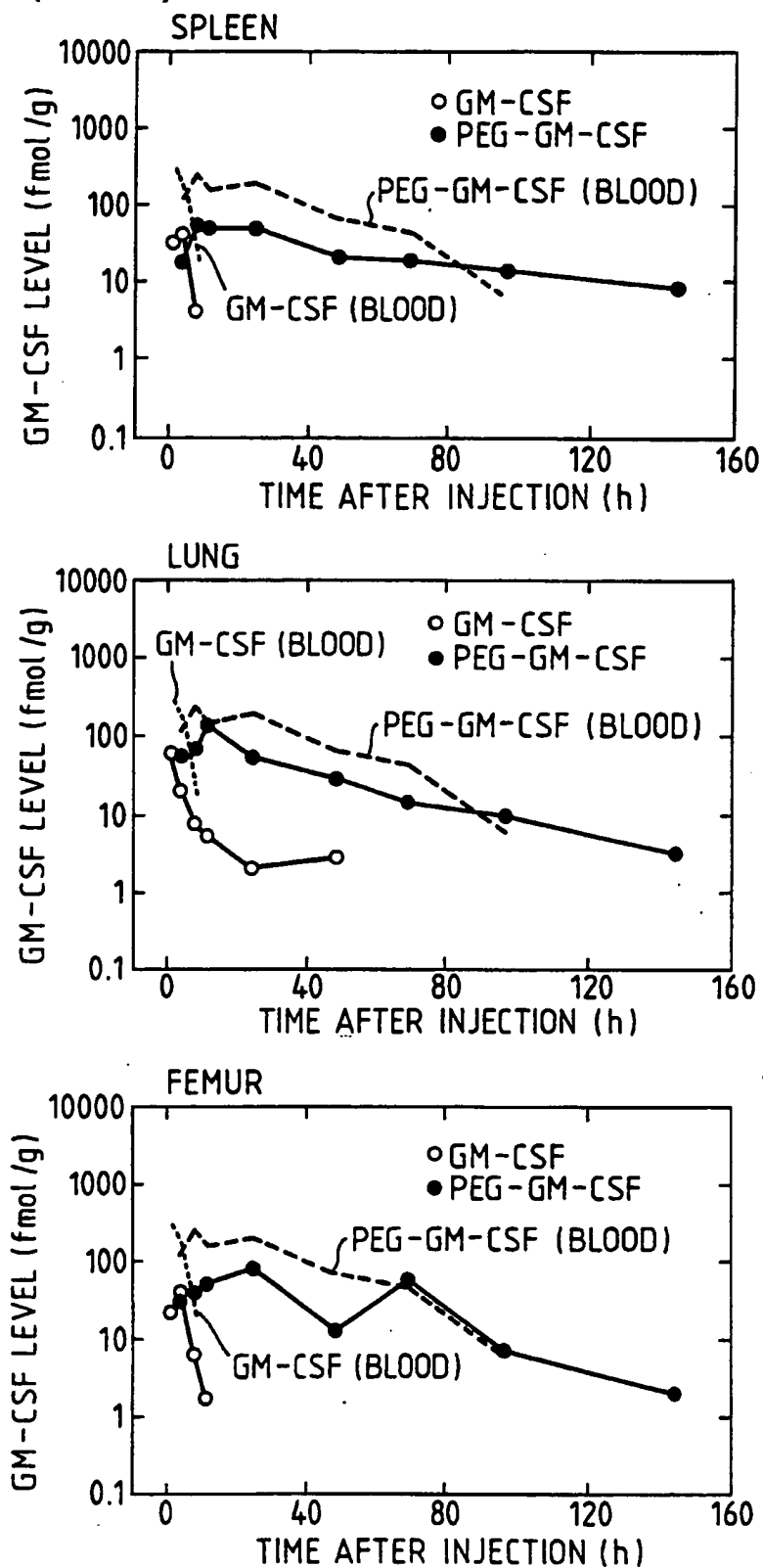
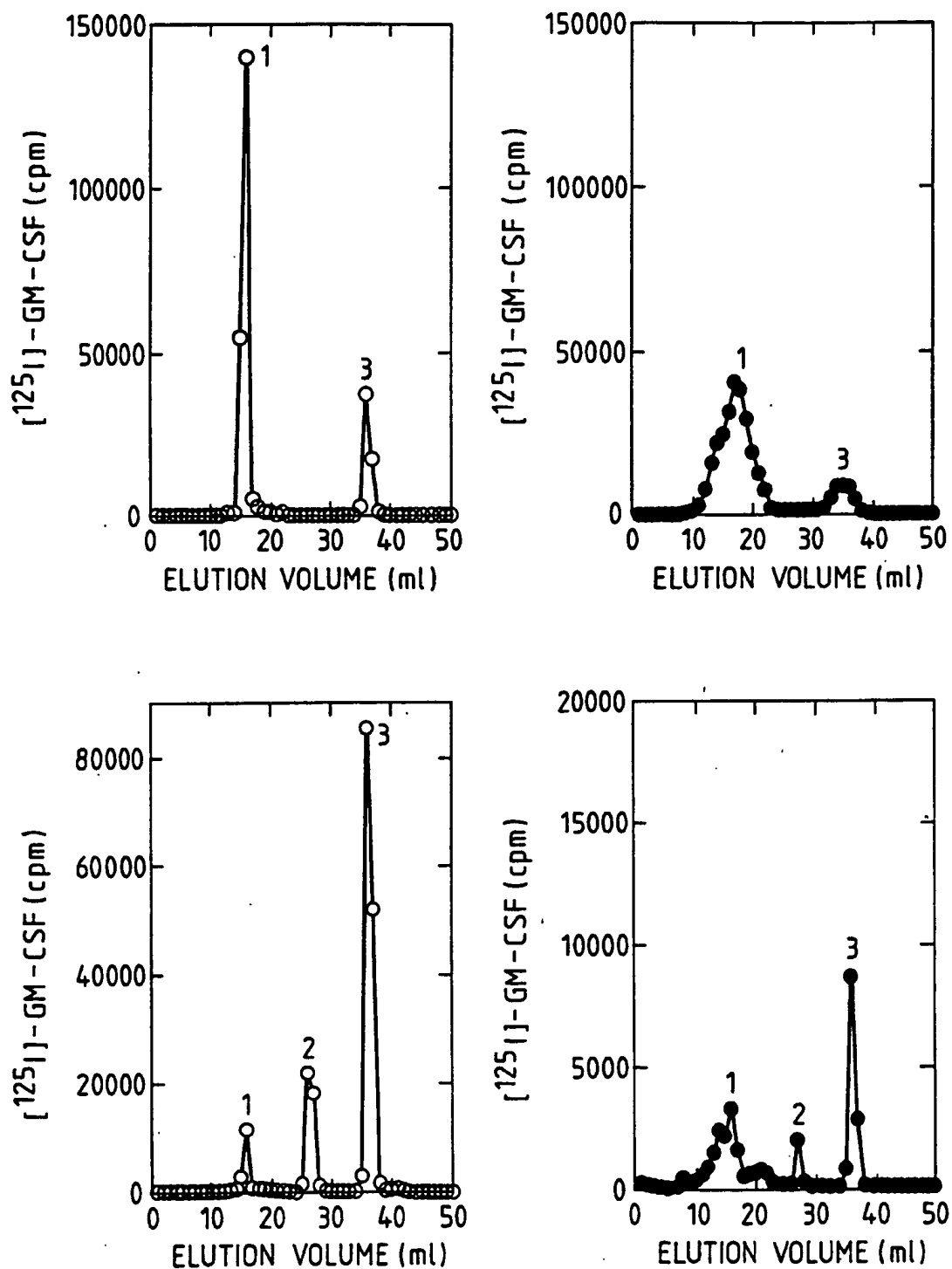


Fig.6b(Cont). 8/14



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Fig.7.



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Fig.8a.

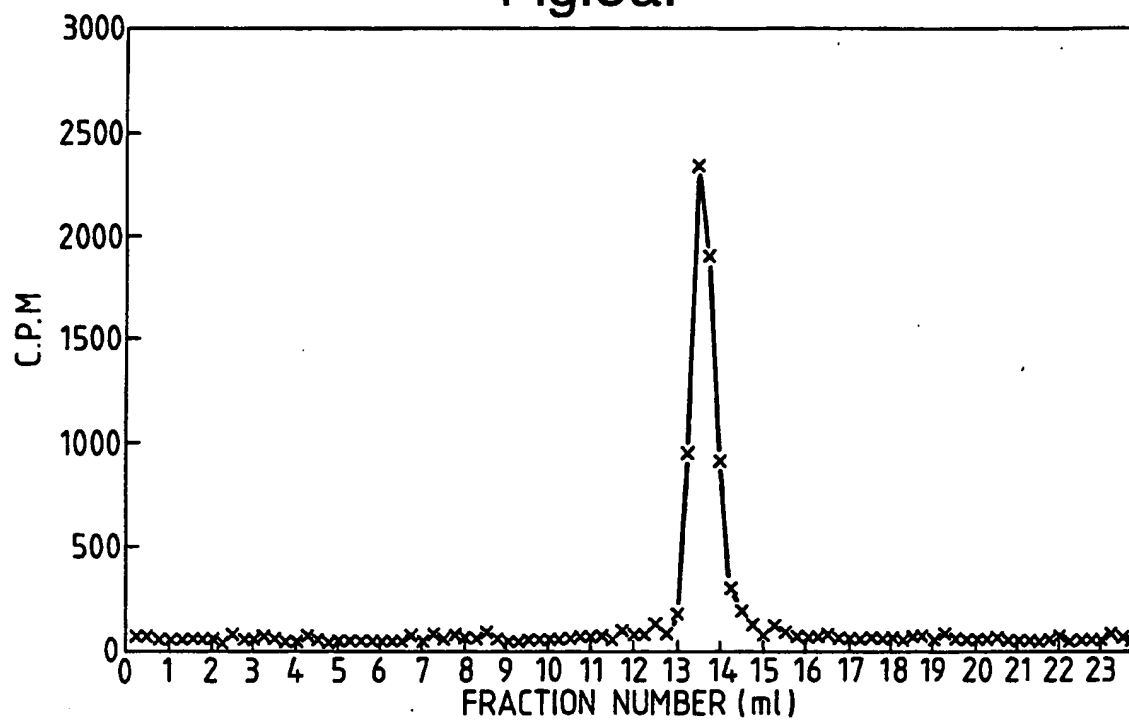
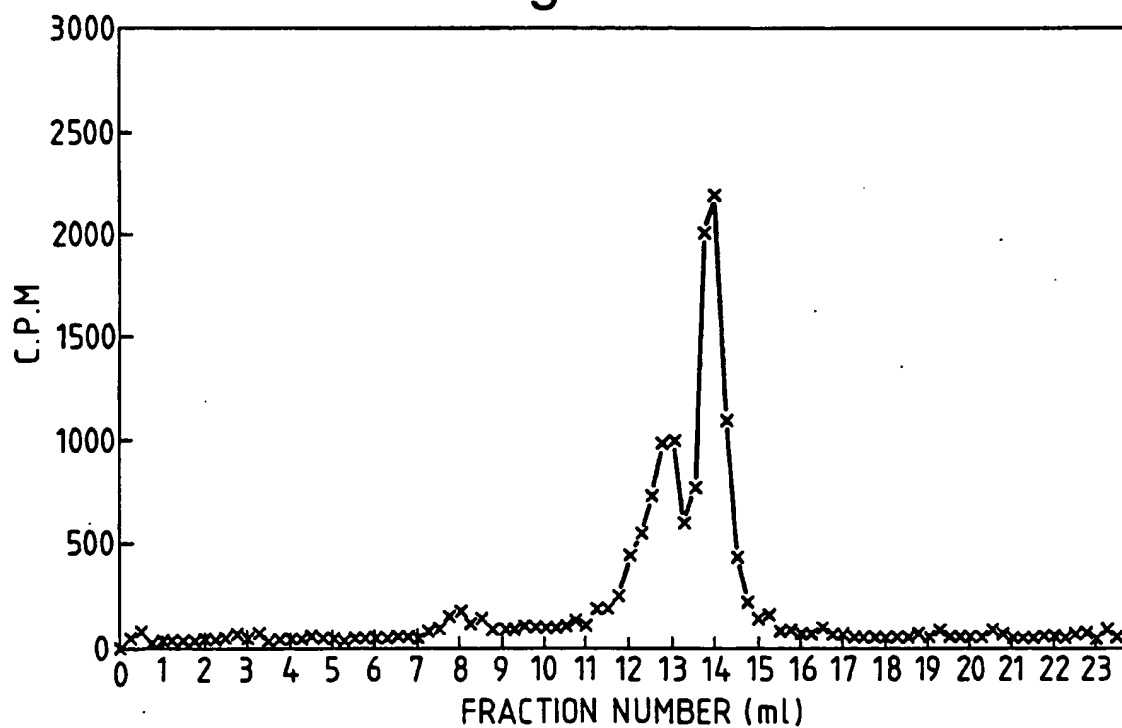


Fig.8b.



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Fig.8c.

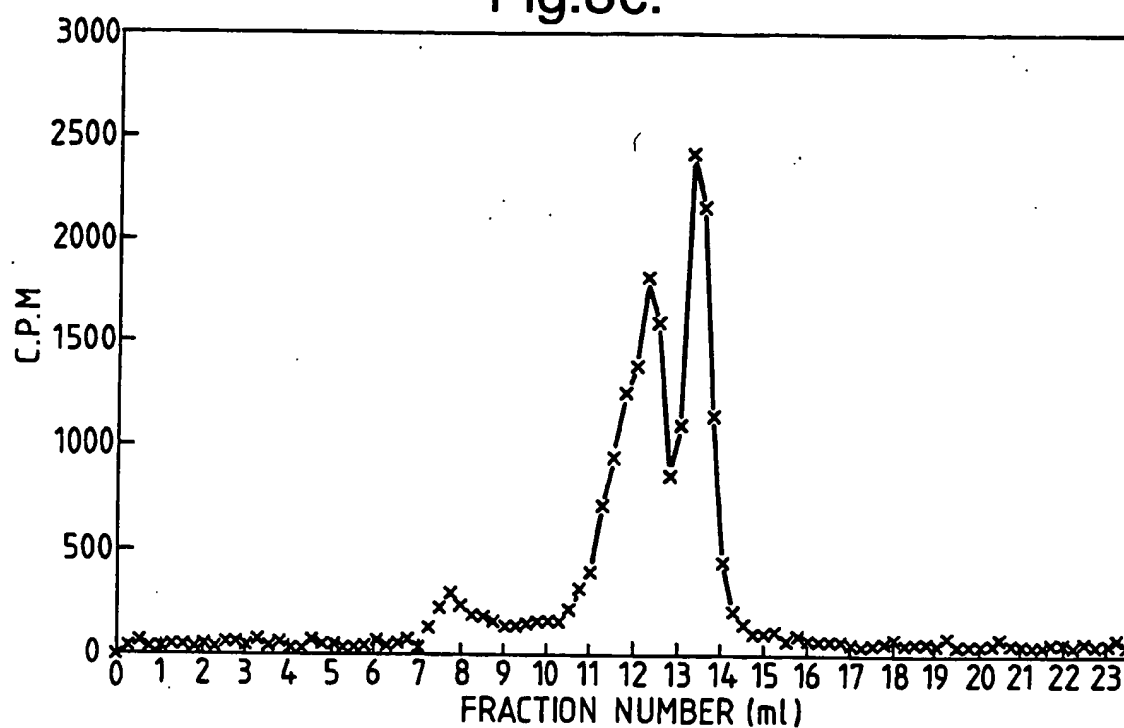
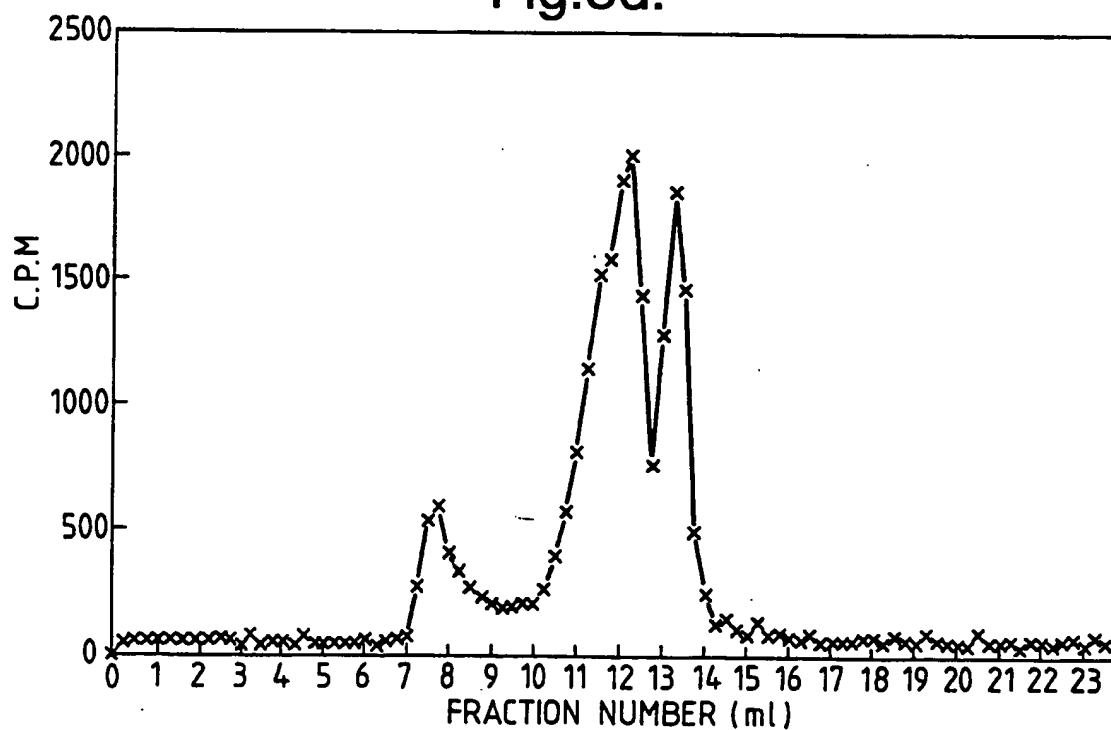


Fig.8d.



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Fig.8e.

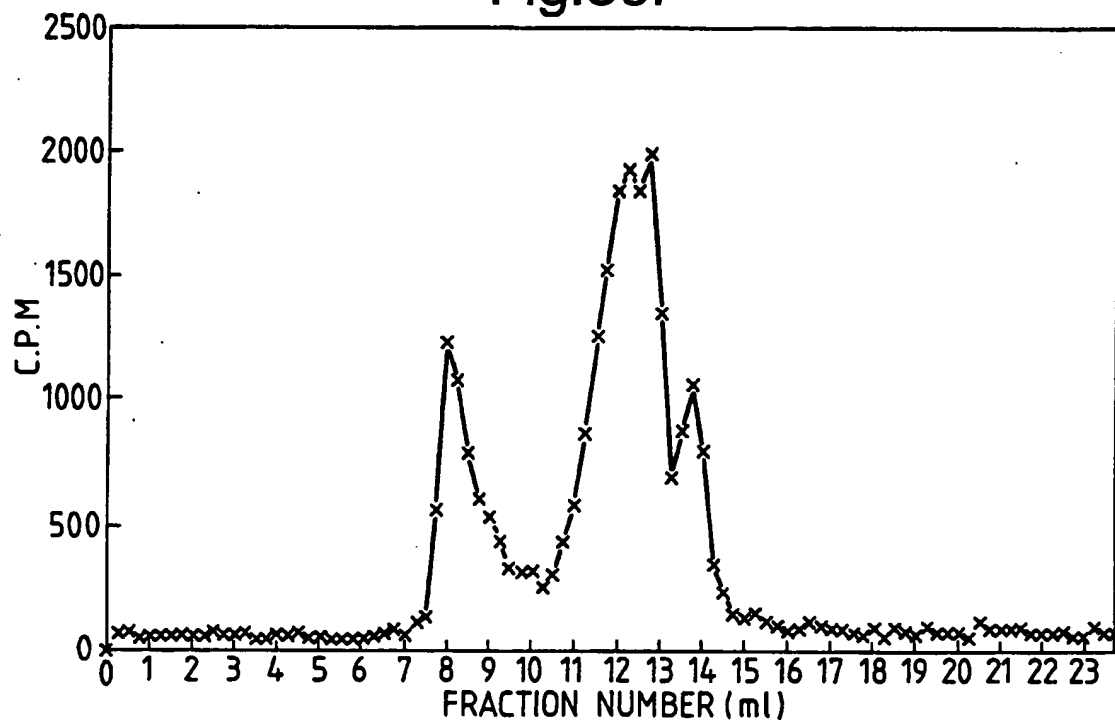
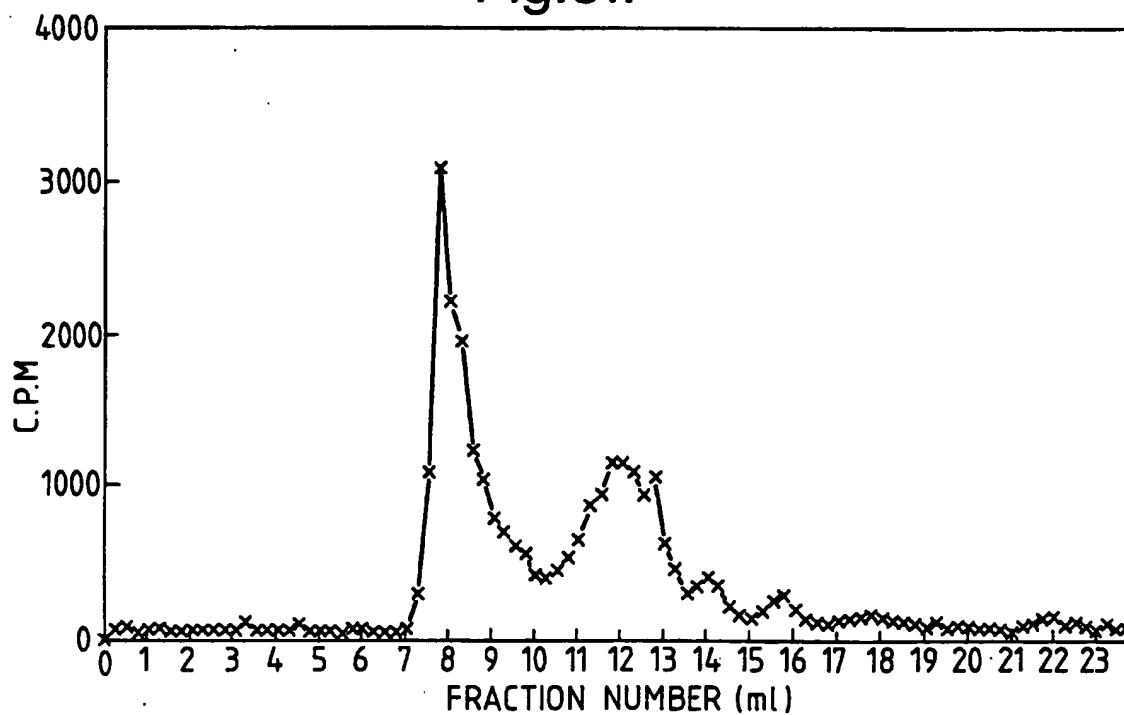
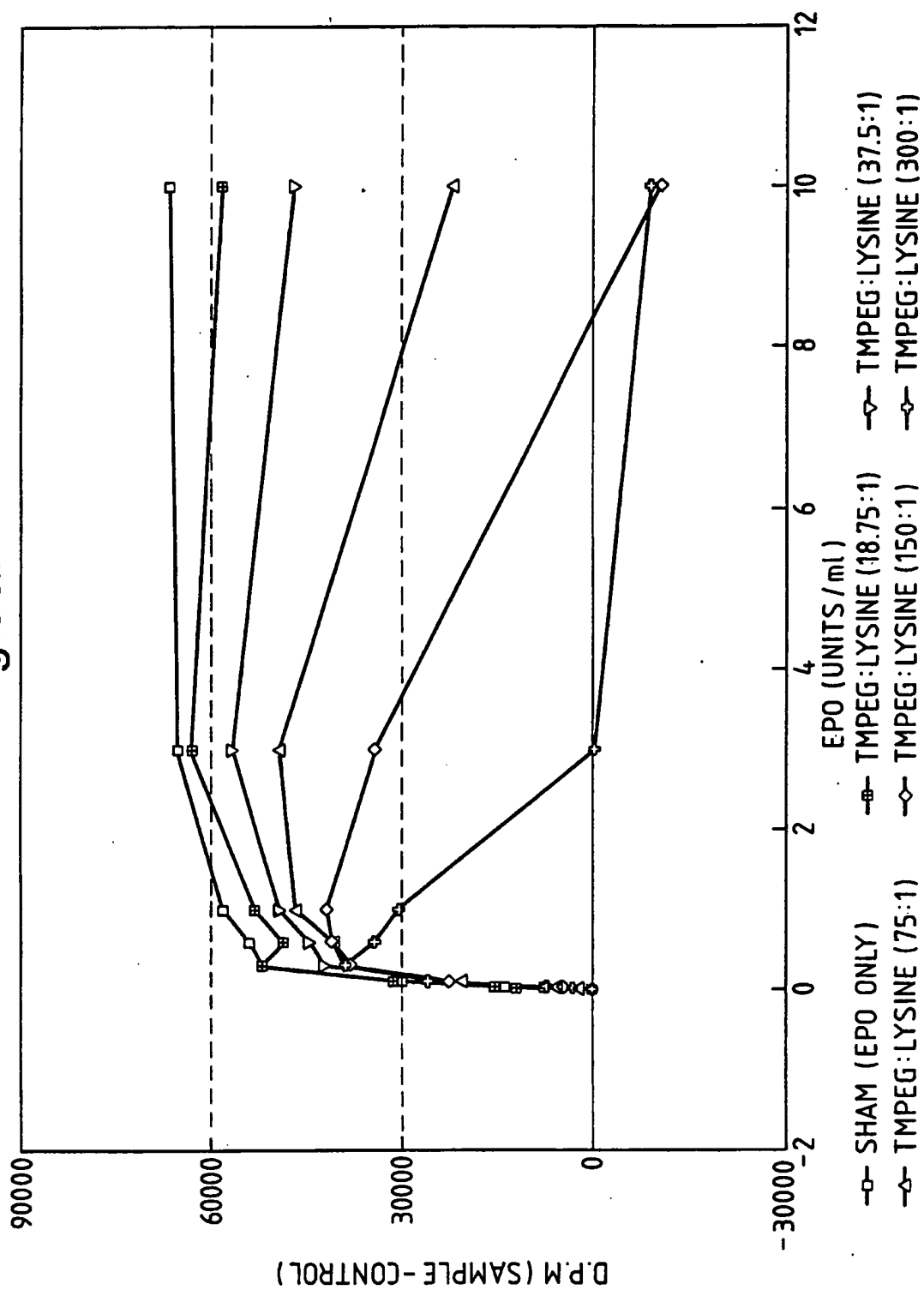


Fig.8f.



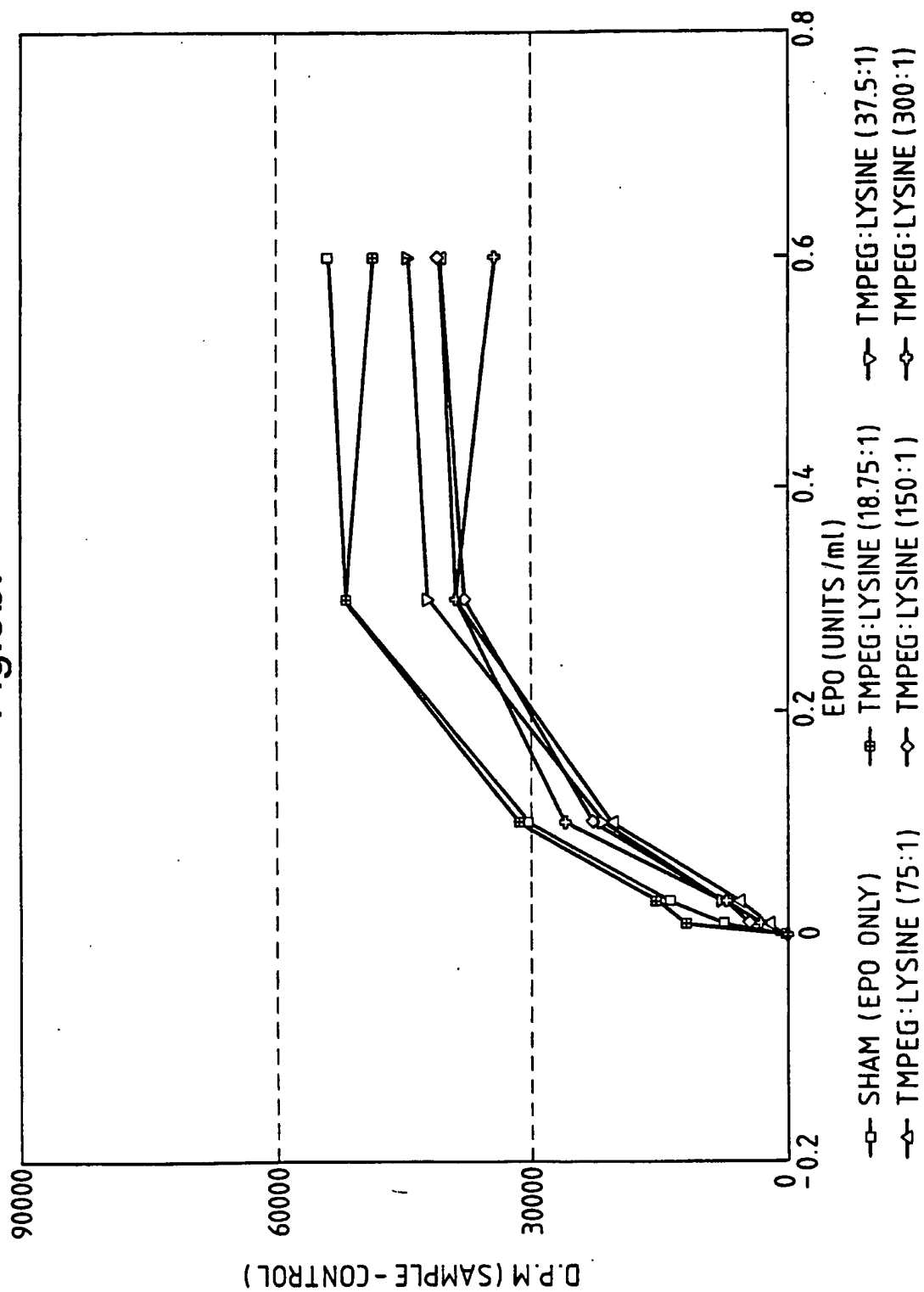
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Fig.9a.



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Fig.9b.



INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/GB 94/01844

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K1/10 C07K1/13 A61K49/00 A61K47/48 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 04606 (ROYAL FREE HOSPITAL SCHOOL OF MEDICINE) 3 May 1990 cited in the application see page 14 - page 16; table II ---	1,2, 4-11, 20-25
X	WO,A,90 04650 (ROYAL FREE HOSPITAL SCHOOL OF MEDICINE) 3 May 1990 cited in the application see page 7 - page 9 ---	1,2, 4-11, 14-18, 20-25
X	WO,A,90 04384 (ROYAL FREE HOSPITAL SCHOOL OF MEDICINE) 3 May 1990 cited in the application see page 3, line 8-9 ---	1,2, 4-11, 14-25
	--- -/--	

☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/GB 94/01844

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EXP.HEMATOL., vol.20, 1992 pages 1028 - 35 F.MALIK ET AL. 'Polyethylene Glycol (PEG)- modified Granulocyte-Macrophage Colony- stimulating Factor (GM-CSF) with conserved Biological Activity' *Materials and Methods* ---	1,2, 4-11, 14-18, 20-25
X	BIOTECHNOL.APPL.BIOCHEM., vol.12, no.2, 1990 pages 119 - 128 C.DELAGO ET AL. 'Coupling of Poly(ethylene glycol) to Albumin under Very Mild Conditions by Activation with Tresyl Chloride: Characterization of the Conjugate by Partitioning in Aqueous Two-Phase Systems' *Materials and Methods* ---	1,2, 5-11, 14-25
X	BIOCHEM.BIOPHYS.ACTA, vol.106, 1991, NETHERLAND pages 77 - 82 J.SENIOR ET AL. 'Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles' see the whole document ---	1,2, 4-11, 14-25
X	BRITISH JOURNAL OF HAEMATOLOGY, vol.82, 1992 pages 654 - 663 C.KNUSLI ET AL. 'Polyethylene glycol (PEG) modification of granulocyte-macrophage colony stimulating factor (GM-CSF) enhances neutrophil priming activity but not colony stimulating activity' see figures 1-4 ---	1,2, 4-11, 14-23
X	ANALYTICAL.BIOCHEM., vol.134, 1983 pages 60 - 72 K.NILSSON ET AL. 'High-Performance Liquid Chromatography on Silica-Bound Alcohol Dehydrogenase' *Materials and Methods* ---	1,2, 4-11, 14-18, 20-25
X	BIOCHEM.SOC.TRANS., vol.16, no.6, 1988 pages 968 - 969 C.DELAGO ET AL. 'Immunoaffinity cell partitioning of erythrocytes' see the whole document -----	1,2, 4-11, 14-18, 20-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Application No

PCT/GB 94/01844

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9004606	03-05-90	EP-A-	0439508	07-08-91
		JP-T-	4501260	05-03-92
		US-A-	5349052	20-09-94

WO-A-9004650	03-05-90	EP-A-	0439502	07-08-91
		JP-T-	4501356	12-03-92

WO-A-9004384	03-05-90	DE-D-	68915001	01-06-94
		DE-T-	68915001	04-08-94
		EP-A-	0445131	11-09-91
		EP-A-	0572049	01-12-93
		JP-T-	4501117	27-02-92

WO9832466

Publication Title:

PEGYLATION PROCESS

Abstract:

The present invention relates to the attachment of a polyethylene glycol (PEG) moiety to a target substrate. Processes for such attachment will be hereinafter referred to as "PEGylation" of the substrate. In particular, the present invention relates to a process for direct covalent PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group in the PEGylation reaction.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 47/48		A1	(11) International Publication Number: WO 98/32466 (43) International Publication Date: 30 July 1998 (30.07.98)
(21) International Application Number: PCT/GB98/00253 (22) International Filing Date: 28 January 1998 (28.01.98) (30) Priority Data: 9701800.6 29 January 1997 (29.01.97) GB 9701804.8 29 January 1997 (29.01.97) GB 9704653.6 6 March 1997 (06.03.97) GB 9708055.0 22 April 1997 (22.04.97) GB (71) Applicant (for all designated States except US): POLYMASC PHARMACEUTICALS PLC [GB/GB]; Fleet Road, London NW3 2EZ (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): FRANCIS, Gillian, Elizabeth [GB/GB]; Summer Cottage, Cane End, Reading, Berkshire RG4 9GH (GB). FISHER, Derek [GB/GB]; 34 Corinium Gate, St. Albans, Hertfordshire AL3 4HY (GB). MALIK, Farooq [GB/GB]; 67 Gracefield Gardens, Streatham, London SW16 2TS (GB). (74) Agent: NACHSHEN, Neil, Jacob; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PEGYLATION PROCESS			
(57) Abstract <p>The present invention relates to the attachment of a polyethylene glycol (PEG) moiety to a target substrate. Processes for such attachment will be hereinafter referred to as "PEGylation" of the substrate. In particular, the present invention relates to a process for direct covalent PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group in the PEGylation reaction.</p>			

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PEGYLATION PROCESS

5

The present invention relates to the attachment of a polyethylene glycol (PEG) moiety to a target substrate. Processes for such attachment will be hereinafter referred to as "PEGylation" of the substrate. In particular, the present invention relates to a process for direct covalent PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group in the PEGylation reaction.

Covalent attachment of PEG to molecules such as proteins or structures such as liposomes is well known to improve their pharmacological and physiological properties.

EP-A-354855 describes a liposome which comprises a PEG-bound phospholipid wherein the PEG moiety is bonded to a phospholipid present in the liposome membrane. This is claimed to provide a reduction in the absorption of proteins to the liposome *in vivo* and hence an increase in its *in vivo* stability.

EP-A-154316 describes a method for chemically modifying lymphokines by attachment of a PEG moiety wherein the PEG is bonded to at least one primary amino group of the lymphokine. This is claimed to result in the delayed clearance of lymphokines when used as drugs and to decrease their antigenicity.

There are many methods for achieving covalent coupling of PEG to substrates. All such methods require the activation of the PEG by attachment of a group usually referred to as an "activating moiety" or by converting a terminal moiety of the PEG into an activating moiety. This is followed by a second step where the PEG couples to the target molecule, usually via a residual portion of the activating moiety which may be referred to as the "coupling moiety".

Examples of known techniques include:

10

Succinimidyl Active Ester Methods: see e.g. US Patent 4,412,989; WO 86/04145; WO 87/00056; EP-A-0 247 860, C. Monfardini, O. Shiavon, P. Caliceti, M. Morpurgo, J. M. Harris, and F. M. Veronese, "A branched monomethoxypoly(ethylene glycol) for protein modification," Bioconjugate Chem., 6,62-69 (1995), Zalipsky, S. et al. (1991) in "Polymeric Drugs and Drug Delivery Systems" (R. L. Dunn & R. M. Ottenbrite, eds.) ACS, Washington, DC, Chapter 10, Zalipsky, S. et al. (1992) Biotechnol. Appl. Biochem. 15:100, Chiu, H.-C. et al. (1993) Bioconjugate Chem. 4:290, Sirokman, G. & Fasman, G. (1993) Protein Sci. 2:1161, Veronese, F. M. et al (1989) J. Controlled Release 10:145, Abuchowski, A. et al (1984) Cancer Biochem. Biophys. 7:175, Joppich, M. & Luisi, P.L. (1979) Macromol. Chem. 180:1381, Klibanov, A. L. et al (1990) FEBS Letters 268:235, Sartore, L. et al (1991) Appl. Biochem. Biotech. 31:213

20

25

Carbonyldiimidazole Method: see e.g. EP-A-0 154 432.

Phenylchloroformate Methods: see e.g. WO 89/06546 and WO 90/15628.

5 PEG-Succinate Mixed Anhydride Methods: see e.g. Ahlstedt et al (1983) Int. Arch. Allergy Appl. Immunol., 71,228-232; Richter and Akerblom (1983) Int. Arch. Allergy Appl. Immunol, 70, 124-131;

Organic Sulphonyl Halide Methods: see e.g. US Patent 4,415,665.

10 PEG-Maleimide and Related Methods: see e.g. Goodson & Katre (1990) Biotechnology, 8, 343-346.

Phenylglyoxal Method: see e.g. EP-A-0 340 741

15 Succinimide Carbonate Method: see e.g. WO 90/13540; WO 91/07190

Cyanogen Bromide Method: see USP 4,301,144

20 Poly-PEG Maleic Acid Anhydride Method: Yoshimoto et al (1987) Biochem. and Biophys. Res. Commun. 148, 876-882.

25 Cyanuric chloride method: Abuchowski, A. van Es, T., Palczuk, N.C., & David, F.F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J. Biol. Chem., 252, 3578-3581.

PEG acetaldehyde methods: Royer, G.P. US 4,002,531 EP-A-0154316. Harris, J. M., Yoshinaga, K. Paley, M.S., & Herati, M. R.

(1989). New activated PEG derivatives for affinity partitioning. In D. Fisher & I.A. Sutherland (Eds) Separations Using Aqueous Phase Systems. Applications in Cell Biology and Biotechnology (pp. 203-210). London Plenum Press.

5

Amine acylation methods (both PEG-COOH and PEG-NH₂): see e.g. EP0072111 and EP 0401384.

10

Vinylsulfone method: M. Morpurgo, F. M. Veronese, D. Kachensky and J. M. Harris, J. Bioconj. Chem., 7, 363-368 (1996).

PEG epoxide methods: Elling, L. & Kula, M-R. (1991) Biotech. Appl. Biochem. 13,354.

15

PEG isocyanate method: R. B. Greenwald. A. Pendri and D. Bolikal, J. Org. Chem., 60, 331-336 (1995).

PEG orthopyridyl-disulphide: C. Woghiren, B. Sharma and S. Stein, Bioconj. Chem., 4,314 (1993).

20

PEG-propionaldehyde: Harris, J.M., Dust, J.M., McGill, Harris, P.A., Edgell, M.J., Sedaghat-Herati, R.M., Karr, L.J., & Donnelly, D.L. (1991). New polyethylene glycols for biomedical applications. Chapter 27 in S.W. Shalaby, C. L. McCormick, & G. B. Butler (Eds.), Water-Soluble Polymers Washington D.C.: American Chemical Society.

25

These methods suffer from one or more of the following defects:
Substantial loss of biological activity (e.g. 20-95% loss of bio-activity) is frequently seen with the cyanuric chloride method:

- 5 Savoca KV, Abuchowski A, van Es T, Davis FF, Palczuk NC (1979),
Biochem Biophys Acta 578: 47-53, Ashihara Y, Kono T, Yamazaki S, Inada
Y (1978) Biochem Biophys Res Commun 83:385-391, Kamisaki Y, Wada H,
Yagura T, Matsushima A, Inada Y (1981) J Pharmacol Exp Ther 216: 410-
414, Wieder K. J. Palczuk NC, van Es T, Davis F F (1979) J Biol Chem
10 254:12579-12587, Nishimura H, Matsushima A, Inada Y (1981) Enzyme
26:49-53 and Pyatak PS, Abuchowski A, Davis FF (1980) Res Commun
Chem Pathol Pharmacol 29:113-127

The coupling of PEG (or other polymers) to proteins (or other target
15 molecules) is, with few exceptions, in a manner which leaves part of the
activating moiety, a coupling moiety, between the PEG and the target
molecule. Of the above methods, only the organic sulphonyl halide methods
and PEG-acetaldehyde methods disclosed in Royer US 4002531 (1977) and
Harris (1989, *ibid*) couple PEG directly without coupling moieties i.e. to
20 produce a "linkerless" PEGylated product. With the exception of some other
PEG acetaldehyde methods where the coupling moiety is ethylene oxide (and
thus indistinguishable from PEG itself) and the direct coupling methods above,
all other coupling methods incorporate a coupling moiety distinct from the
polymer and the target and are thus regarded as "indirect" coupling methods.

25

The incorporation of a coupling moiety generates further problems
depending on the nature of the coupling moiety, thus

- (i) some coupling moieties provide targets for enzymatic cleavage or hydrolysis (see below);
- 5 (ii) some coupling moieties provide an immunogenic/antigenic group (e.g. the triazine ring of the cyanuric chloride method or the succinyl group of the succinimidyl succinate method and PEG succinate mixed anhydride method);
- 10 (iii) some coupling moieties are potentially toxic or are themselves of unknown toxicity but derived from a compound known to be toxic (e.g. the triazine ring of the cyanuric chloride method and reagents in the phenylchloroformate method); and
- 15 (iv) some coupling moieties provide reactive groups capable of linking further molecules to the PEG-target construct via the coupling moiety (e.g. the triazine ring of the cyanuric chloride method, Leonard, M. *et al.*, Tetrahedron, 40: 1585 (1984)) and,
- 20 (v) Some coupling groups alter surface charge at the site of attachment of the polymer.
- 25 Coupling in some instances is thus via an unstable bond liable to be cleaved by enzymes present in serum, plasma, cells or other biological materials or by procedures applied to the PEG-target product. This has two possible deleterious consequences,

- (i) the PEG-target construct is degraded enzymatically or by the conditions required for subsequent reaction steps; the former occurs particularly with methods generating ester bonds and probably also with amide bonds; and
- 5 (ii) removal of the PEG moiety alters the target molecule; this occurs with some succinimidyl active ester and mixed anhydride methods,
- 10 and either or both of these can occur.

Many of the above methods recommend long coupling times and/or a non physiological pH for the PEGylation reaction, thus rendering some target molecules less active or inactive (cf. the cyanuric chloride, phenylchloroformate, acetaldehyde and propionaldehyde methods).

15

Many of these methods use activated PEG species and/or produce co-products which are toxic in a wide range of bioassays and which are potentially toxic *in vivo* if not separated from the product (e.g. the phenylchloroformate, cyanuric chloride methods).

20

Some methods are unsuitable for use in aqueous solution, thus limiting the target molecules to those which will tolerate non-aqueous conditions (cf. the organic sulphonyl halide method using trifluoromethanesulphonyl chloride).

25

Some of the activated PEG-target constructs are unstable, for instance being subject to hydrolysis during either the activation or coupling reactions (cf. the phenylchloroformate method). For example, PEG acetaldehyde is

sensitive to decomposition under basic conditions and can give inconsistent results.

Ouchi T., et al [(1987) J. Macromol. Sci. Chem. A24 1011-1032]
5 discusses the PEGylation of 5-fluorouracil with various methoxy-PEG
derivatives to generate methoxy-PEG ether, ester or amide-linked constructs.
The preparation of methoxy-PEG-ether-5-fluorouracil from a methoxy-PEG-
Br derivative in chlorobenzene using tetra-n-butylammonium bromide as a
phase-transfer catalyst is described. None of the methoxy-PEG-ether-5-
10 fluorouracil derivatives thus produced showed bioactivity (i.e. anti-tumour
activity).

Zheng Hu et al (1987) Acta Pharmaceutical Sinica, 22 (8) 637 - 640
discusses the synthesis of PEG-estrogen compounds from chlorinated
15 polyethylene in non-aqueous solvents using the Williamson reaction.

Probably the most advantageous PEGylation method employed hitherto
is the TMPEG method, mentioned in WOA-90/04606, which comprises
activation of monomethoxy PEG ("MPEG") with 2,2,2-
20 trifluoroethanesulphonyl chloride (tresyl chloride) to produce tresyl MPEG
("TMPEG") which is subsequently reacted with a target protein molecule to
produce monomethoxy PEGylated products. At physiological pH the TMPEG
method is a "direct" coupling method in that the PEG moiety is coupled
directly to the target substrate without a coupling or linker moiety. A similar
25 technique is described in WO 90/04650 for coupling monomethoxy PEG
moieties to DNA/protein complexes.

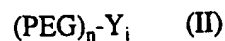
It is also known that the use of TMPEG as the activated PEG for use
in PEGylation can, particularly at very high pH, result in the elimination of

HF by an alternate pathway. This alternative elimination pathway may occur for example when reacting TMPEG with a protein and involves the elimination of HF which converts TMPEG into an intermediate alkene followed by hydration and further elimination of HF to create the acyl fluoride which is converted to the α -sulphonate acid via further hydrolysis. The alkene and acyl fluoride MPEG derivatives can react with target molecules to form a sulphonate amide derivative.

It is clearly desirable to develop a functionalised PEG which is simple and cheap to prepare, which can be used to PEGylate a wide range of potential substrates, which generates a linkerless or directly coupled PEGylated substrate, is capable of pegylating a substrate in both aqueous and non-aqueous solvents and which does not result in any of the undesirable side effects listed above. It is also desirable to have a PEGylation process which functions rapidly under physiological conditions since this is critical for retention of biological activity in the PEGylation of many proteins.

Hence there is provided according to the present invention a process for the PEGylation of a substrate comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group and the PEG is bound directly to the substrate.

In a preferred embodiment of the present invention the halogenated PEG for use in the process of the invention is of general formula I or general formula II.



wherein:

In formula I PEG is a bivalent group of formula $-(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2-$, where m is equal or greater than 1, derived from a polyethylene glycol;

5 X is a halogen atom, a blocking group or an activating group capable of coupling the PEG moiety to another moiety; Y is a halogen; n represents the number of PEG termini and n is equal or greater than 2; i is equal or less than n; and i/n PEG termini are substituted by Y in compounds of formula II.

10 Halogenated PEGs of Formula I may be monofunctional, homobifunctional, or heterobifunctional activated PEGs i.e. a halogenated PEG of formula I may have two terminal halogens (X and Y are halogens which may be the same or different), or when only one terminal halogen is present the other terminal group X may be either a blocking group or an
15 activating group. Halogenated PEGs of Formula II may be of branched, cruciform or stellate structure. In preferred embodiments of the present invention, X is a blocking group selected from methyl, t-butyl and benzyl ethers.

20 In further preferred embodiments of the present invention, X is an activating group having an atom that is susceptible to nucleophilic attack or is capable of rendering the terminal carbon atom of the PEG susceptible to nucleophilic attack or equivalent alternative substitution and is preferably a sulphonate ester, a substituted triazine, a N-hydroxysuccinimide active ester, an
25 anhydride, a substituted phenyl carbonate, oxycarbonylimidazole, a maleimide, an aldehyde, a glyoxal, carboxylate, a vinyl sulphone, an epoxide, an isocyanate, a disulphide, an acrylate, an allyl ether, a silane or a cyanate ester. More preferably X is an activating group selected from

- 2,2,2-trifluoroethanesulphonate,
pentafluorobenzenesulphonate,
fluorosulphonate,
2,4,5-trifluorobenzenesulphonate,
5 2,4-difluorobenzenesulphonate,
2-chloro-4-fluorobenzenesulphonate,
3-chloro-4-fluorobenzenesulphonate,
4-amino-3-chlorobenzenesulphonate,
4-amino-3-fluorobenzenesulphonate,
10 o-trifluoromethylbenzenesulphonate,
m-trifluoromethylbenzenesulphonate,
p-trifluoromethylbenzenesulphonate,
2-trifluoromethoxybenzenesulphonate,
4-trifluoromethoxybenzenesulphonate,
15 5-fluoro-2-methylbenzenesulphonate,
4,6-dichlorotriazine,
6-chlorotriazine,
N-hydroxysuccinimidyl succinate,
N-hydroxysuccinimidyl glutarate,
20 N-hydroxysuccinimidyl succinamide,
N-hydroxysuccinimidylalkanedioicamides,
N-hydroxysuccinimidyl derivatives of carboxymethylated polymers,
succinimidylcarbonate,
N-hydroxysuccinimidyl esters of amino acids,
25 succinate mixed anhydride,
succinic anhydride,
trichlorophenyl carbonate,
nitrophenyl carbonate,
maleimide,

N-substituted maleimide,
acetaldehyde,
propionaldehyde and chemically equivalent sulphur analogues,
glyoxal,
5 phenylglyoxal,
acrylate,
methacrylate.

Preferred halogens for the groups X and Y include chlorine, bromine
10 and iodine. Chlorine is most preferred.

In the most preferred embodiments of the present invention, the
halogenated PEG is one of

- 15 - monomethoxy PEG-Cl
- monomethoxy PEG-Br
- monomethoxy PEG-I
- Cl-PEG-Cl
- Br-PEG-Br
- 20 - I-PEG-I

Although some halogenated PEGs are known compounds, it is
particularly surprising that they have utility as PEG derivatives suitable for
direct use in a PEGylation reaction. It was previously believed that halogens
25 would be of vastly inferior reactivity to known leaving groups such as tosylate
or tresylate. For example McMurry J. in "Organic Chemistry" 4th Ed. (1996)
cites chlorine as having 300 times less reactivity than tosylate as a leaving
group. When considering that tresylate is described as having 100 fold greater
reactivity than tosylate (March J. Advanced Organic Chemistry Reactions,

Mechanisms and Structure, 4th Ed. [1992]), it may be concluded that chlorine would be expected to have 30,000 fold less reactivity than tresylate.

Halogenated PEGs may be synthesised by methods well known in the art. PEG may be synthesised or purchased commercially and then derivatised with halogen, activating groups or blocking groups, as required, using methods disclosed in e.g. Bayer E. et al, Polymer Bulletin 8, 585 - 592 (1982); Zalipsky, S et al, Eur. Polym. J. Vol 19 No. 12 pp 1177-1183 (1983); Buckmann A. F., Morr. M and Johansson G., Makromol. Chem. 182, 1379-1384 (1981); Harris, J.M. J. Macromol. Sci., Rev. Polym. Chem. Phys. C25(3) 325-373 (1985); Harris, J.M., Struck, E. C., Case, M. G., et al. J. Poly. Sci, Poly. Chem. Ed. 22, 341-352 (1984); Zalipsky, S. & Lee, C. in Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical applications (ed Harris, J. M.) 347-370 (Plenum Press, New York, 1992); and as reviewed in Zalipsky S. Bioconjugate Chem. (1995) 6 150-165 where MPEG-Cl was used to prepare activated PEGs which were subsequently linked to substrates.

Multi-halogenated PEGs can be constructed using either naturally branched PEGs, such as the cruciform PEG found in some preparations of high molecular weight PEGs, or from proprietary multibranched PEGs known as "star" PEGs. Derivatisation of the free PEG termini with halogen is achieved as for halogenated PEGs above.

Reaction conditions for the process of the present invention will clearly depend upon the nature of X, Y and of the substrate.

As indicated above, the PEG moieties of the halogenated PEG's used in accordance with the invention, may desirably be derived from commercially available PEGs. These materials are generally characterised by their number

and weight average molecular weight. For example, PEG-5000 is a polyethylene glycol having a number average molecular weight of about 5000. The size of the PEG moiety to be attached to the target substrate will usually be chosen according to the nature of the substrate and how its properties are desired to be modified by the attachment of the PEG moiety. For example, if the target substrate is a liposome for administration to an animal and it is desired to increase the circulation half life of the liposome after administration, a PEG of molecular weight 1000 to 5000 may be selected. It should be noted, however, that the process of the present invention is generally applicable to the attachment of PEG moieties of any size to target substrates.

PEGylated substrates generated according to the present invention particularly include those which do not lose their bioactivity relative to the unPEGylated substrate. Thus PEGylation according to the present invention may maintain or increase the specific activity of a substrate or it may increase the *in vivo* half-life of a substrate which has had its specific activity decreased, maintained or increased by PEGylation. Additionally PEGylation according to the present invention may differentially modify the specific activity of pleiotropic substrates such as certain proteins.

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The term "substrate" as used herein is intended to include any molecule, macromolecule or structure which is capable of being covalently attached to a PEG moiety and which thereby may have its chemical, biological, physiological or physical properties modified. It is not intended to encompass molecules which when reacted with halogenated PEG merely produce a further activated PEG derivative which is to be used as an intermediate to couple the PEG moiety to another substrate. The substrate is not a steroid.

Suitable substrates to which PEG can be attached in accordance with the present invention include materials having biological activity which are useful in, for instance diagnosis or therapy and which are all well known to those skilled in the art. They all contain at least one group capable of reacting with the halogenated PEG. Examples of such reactive groups include primary, secondary and tertiary amino groups, thiol groups and aromatic hydroxy groups.

More specifically, substrates for use according to the present invention include proteins, peptides, amino acids and their derivatives such as antibodies and fragments thereof; cytokines and derivatives or fragments thereof, for example, the interleukins (IL) and especially the IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and IL-12 subtypes thereof; colony stimulating factors, for example granulocyte-macrophage colony stimulating factor, granulocyte-colony stimulating factor (alpha and beta forms), macrophage colony stimulating factor (also known as CSF-1); haemopoietins, for example erythropoietin, haemopoietin-alpha and kit-ligand (also known as stem cell factor or Steel factor); interferons (IFNS), for example IFNalpha, IFNbeta and IFNgamma; growth factors and bifunctional growth modulators, for example epidermal growth factor, platelet derived growth factor, transforming growth factor (alpha and beta forms), amphiregulin, somatomedin-C, bone growth factor, fibroblast growth factors, insulin-like growth factors, heparin binding growth factors and tumour growth factors; differentiation factors and the like, for example macrophage differentiating factor, differentiation inducing factor (DIF) and leukaemia inhibitory factor; activating factors, for example platelet activating factor and macrophage activation factor; coagulation factors such as fibrinolytic/anticoagulant agents including heparin and proteases and their pro-factors, for example clotting factors VII, VIII, IX, X, XI and XII,

antithrombin III, protein C, protein S, streptokinase, urokinase, prourokinase, tissue plasminogen activator, fibrinogen and hirudin; peptide hormones, for example insulin, growth hormone, gonadotrophins, follicle stimulating hormone, leutenising hormone, growth hormone releasing hormone and
5 calcitonin; enzymes such as superoxide dismutase, glucocerebrosidase, asparaginase and adenosine deaminase; vaccines, for example hepatitis-B vaccine, malaria vaccine, melanoma vaccine and HIV-1 vaccine; transcription factors and transcriptional modulators; carbohydrates, glycosoaminoglycans, glycoproteins and polysaccharides; lipids, for example phosphatidyl-
10 ethanolamine, phosphatidylserine and derivatives thereof; sphingosine; and derivatives thereof; nucleotides, nucleosides, heterocyclic bases, DNA, RNA, synthetic and non-synthetic oligonucleotides including those with nuclease resistant backbones; vitamins; antibiotics including lantibiotics; bacteristatic and bactericidal agents; antifungal, anthelmintic and other agents effective
15 against infective agents including unicellular pathogens; small effector molecules such as noradrenalin, alpha adrenergic receptor ligands, dopamine receptor ligands, histamine receptor ligands, GABA/benzodiazepine receptor ligands, serotonin receptor ligands, leukotrienes and triiodothyronine; cytotoxic agents such as doxorubicin, methotrexate and derivatives thereof.

20

The substrate may also be part of a larger multi-molecular structure. These include cells or parts thereof, for instance erythrocytes, erythrocyte "ghosts" and leukocytes, viruses, unicellular organisms, liposomes such as multilamellar vesicles and unilamellar vesicles, micelles and micelle-like
25 structures, and aggregates, microemulsions, coacervates, emulsions and suspensions of the foregoing. The substrate may also be a surface on a device such as a catheter, stent, contact lens or artificial valve.

It will be appreciated that when the substrate is part of such a structure there will generally be many reactive groups in each structure; treatment according to the invention may therefore produce a structure bearing many PEG moieties. When the PEG is bi- or multi-valent, reaction with a multimolecular substrate may result in intermolecular cross-linking by the PEG between molecules of the same target structure and/or between molecules of different target structures as well as intramolecular bonding of the PEG to more than one position on the same molecule of a target structure.

Substrates lacking a reactive group may be modified so as to create one or more reactive groups; this is within the ability of those skilled in the art and can be achieved by well-known techniques.

Some substrates (e.g. RNA and single stranded DNA) pose special problems because they may provide too many reactive groups to which the PEG would attach in a standard reaction. Therefore, if desired, some groups may be temporarily protected by involvement in an appropriate conformation precluding nucleophilic attack on the halogenated PEG, as for example by the hydrogen bonding associated with base pairing of DNA (see below).

The term "blocking group" as used herein is intended to imply a moiety which when covalently bound to a PEG terminus, is capable of preventing the attachment of an activating group to that terminus during the activation process.

An embodiment of the present process involves site-specific modification of DNA, RNA and synthetic oligonucleotide targets (or of any molecule containing an amino or other reactive group which can participate in interactions such as hydrogen bonding with another molecule or compound)

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by precluding nucleophilic attack on the halogenated PEG species by reactive groups on the target. The bases adenine (A), cytosine (C) and guanine (G) [but not uracil (U) or thymine (T)] provide suitable targets in DNA, RNA and synthetic oligonucleotides for modification with PEG moieties according to the invention and thus these are special targets with the problem that there may be too many available reactive groups to which the polymer can be attached. By using various restriction fragment DNA cleavage sites as a model system, selected bases A, C or G can be modified by the expedient of leaving short stretches (e.g. 2-4 bases) single stranded. Adenine bases appear to be the most susceptible to such modification. Blunt ended double stranded DNA is not readily coupled under the conditions described, indicating that hydrogen bonding between base pairs is sufficient to preclude interaction of the amino groups of A, C and G bases with the activated PEG.

Site-specific DNA modification by polymer can be achieved by the expedient of including one or more A, C or G bases in a short single stranded section of DNA by appropriate restriction enzyme digestion or by hybridising oligonucleotides of dissimilar lengths with the DNA to protect bases which are not to be modified or by exploiting the natural strand asymmetry of polymerise chain reaction products which have a one base-pair overhand, or by exploiting localised regions of single strandedness achieved by natural or artificial localised melting of the double helix. The reaction of methoxyPEGBr with 5-FU is not included within the present invention.

Also provided according to the present invention are products produced by a process of the present invention; the use of such products in medical therapy; pharmaceutical formulations comprising products of the present invention; the use of a product or a pharmaceutical formulation of the present invention in the manufacture of a medicament for use in medical

therapy; and methods of medical therapy comprising the administration of a product or a pharmaceutical formulation of the present invention, to a patient.

The term "medical therapy" as used herein includes therapeutic,
5 diagnostic and prophylactic regimes.

Non-limiting examples of the invention will now be described with reference to the accompanying Figures, which show:-

10 **FIGURE 1** Reverse phase liquid chromatography elution profiles of a) MPEG-Cl; b) MPEG and c) a mixture of MPEG-Cl and MPEG.

15 **FIGURE 2** a) A representative elution profile of the reaction products of the PEGylation of lysozyme using MPEG-Cl, separated on a Superose 12 column.
b) A further example of a similar experiment to that shown in Figure 2a

20 **FIGURE 3** A representative elution profile of reaction products of the PEGylation of lysozyme using TMPEG, separated on a Superose 12 column.

25 **FIGURE 4** Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme by MPEG-Cl at alkaline pH.

FIGURE 5 Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme by MPEG-Cl at a) 4°C for 21min and b) 4°C for 72.5h.

FIGURE 6 Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme using TMPEG at 3°C, 15.5°C and 22.5°C.

5

FIGURE 7 Reverse phase liquid chromatography of polymer species (MPEG, MPEG-Cl and TMPEG in peaks 1-3 respectively) formed using the procedure set out in example 5.

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FIGURE 8 The elution profile from a Superose 12 column (using a computerised FPLC) of the reaction products of the PEGylation of lysozyme using MPEG-Cl produced by the method of example 5.

15

FIGURE 9 The elution profile from a Superose 12 column (using a computerised FPLC) when the amount of TMPEG is equivalent to 10% of the total activated polymer used in Figure 8 above (i.e. similar to the level of contaminating TMPEG in the sample used for PEGylation in Figure 8).

20

FIGURE 10 The elution profile from a Superose 12 column (using a computerised FPLC) of the reaction products of the PEGylation reaction of lysozyme described in Example 6; i.e. using MPEG-Cl, produced by the method of Example 5, from which traces of TMPEG had been removed by prolonged hydrolysis as set out in Example 6.

25

FIGURE 11 Dose response curves of PEGylated GM-CSF produced using MPEG-Cl synthesised as set out in Example 5 and sham-treated

controls exposed to MPEG. The results shown in panels a, b and c are from three independent experiments.

5 **FIGURE 12** Dose response curves of PEGylated EPO produced using MPEG-Cl synthesised as set out in Example 1 and a sham-treated control exposed to MPEG.

10 **FIGURE 13** Dose response curves of PEGylated EPO produced using MPEG-Cl synthesised as set out in Example 5 and sham treated controls exposed to MPEG. Panels a-c show three independent experiments.

15 **FIGURE 14** Acid elimination by MPEG-Cl at pH 7 and Fluoride measurements (for comparison with Figure 15).

FIGURE 15 Fluoride and acid elimination by TMPEG at pH 7: a) TMPEG produced by the method of [WO 95/06058]; b,c) TMPEG produced by two alternate manufacturing procedures.

20 **FIGURE 16** Acid elimination for MPEG-Cl at pH 9 and Fluoride measurements for comparison with Figure 17.

FIGURE 17 Fluoride and acid elimination for TMPEG at pH 9.

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EXAMPLES

EXAMPLE 1 Preparation and characterisation of MPEG chloride

MPEG-chloride was synthesised as described by a modification of the method of *Bayer et al (1982)*. Methoxypolyethylene glycol (Molecular weight 5000, Shearwater Polymers Inc) (5g) was refluxed on an oil bath with twice re-distilled thionyl chloride (10ml) for 15h under nitrogen. The thionyl chloride
5 was removed by distillation. 5ml of dry toluene (molecular sieve, 3A, BDH) was added and distilled off. 5ml of dry dichloromethane (molecular sieve, 3A, BDH) was then added and distilled off. The residue was dissolved in 20 ml of dry dichloromethane and 200ml of dry ether was added at room temperature and the mixture stirred in an ice bath. After storage overnight at -20°C, the white solid
10 was removed by filtration, redissolved in 20 ml of dry dichloromethane and reprecipitated with 200ml of dry ether. The precipitate was removed by filtration and dried in vacuo (*Yield 3.8 g*).

The product showed a single peak on reverse phase liquid
15 chromatography that was distinct from the single peak shown by the MPEG starting material (Figures 1a-c), indicating complete derivatisation of the starting MPEG to MPEG-Cl.

All the samples were analysed as 0.2% w/v solutions in 30%
20 CH₃CN/70%H₂O on a reverse phase column PLRP-S 100A 5μ from Polymer Laboratories, using 30 to 100% CH₃CN gradient. The elution conditions were as follows: using a flow rate of 0.5ml/minute, 30-50% CH₃CN over 20 minutes, then 50-100% CH₃CN over 2 minutes, held at 100% CH₃CN for 3 minutes under isocratic conditions, reverted back to 30% CH₃CN over 1 minute, and
25 finally held at 30% CH₃CN for 5 minutes. The sample was injected via a 20μl loading loop. An evaporative Mass Detector (PL-EMD 960; Polymer Laboratories) at 85°C with gas flow at 5.5litres/minute, was used to monitor the samples.

Figure 1a shows a typical elution profile of MPEG-Cl. A major peak is seen eluting with a retention time at 16.3 min (range 15.9-16.3 min in three experiments). Figure 1b shows a typical elution profile of MPEG-5K, the starting material for preparation of MPEG-Cl. A single peak is seen eluting with a retention time of circa 13.7 min (range 13.0-13.7 in five experiments). Figure 1c shows the elution profile of a mixture of the MPEG-Cl and MPEG-5K samples, by pooling equal volumes of MPEG-Cl and MPEG-5K used to produce the profiles in Figures 1a and 1b above. Two well resolved peaks are seen with retention times corresponding closely to those obtained in Figures 1a and 1b.

¹H nmr of the MPEG-Cl in d₆-DMSO showed an absence of -OH signal (which is typically seen around at 4.56ppm for MPEG in DMSO). A complex multiplet centred around 3.7 ppm was consistent with literature values for O-CH₂-CH₂-Cl.

Example 2 PEGylation of lysozyme

51.25mg of activated MPEG-Cl as prepared in (Example 1) was reacted with 0.466ml of 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at 27°C. The initial polymer concentration was 110 mg/ml. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 50ml of PBS buffer at a flow rate of 0.3ml/min with continuous UV monitoring (214nm) at the outlet. The sensitivity of the UV detector was set at 0.5 absorbance units. The unreacted lysozyme eluted at circa 19.52ml and the PEGylated lysozyme conjugates eluted at circa 10.79, 13.02, 14.94 (Figure 2a). The chromatogram indicates that significant reaction occurred between the MPEG-Cl and lysozyme under the

above conditions. This reactivity rate was reproduced in further independent experiments.

For example, 48.15mg of MPEG-Cl was reacted with 0.438ml of
5 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at
28°C. The initial polymer concentration was 109 mg/mL. 100ul of the reaction
mixture was diluted with 400ul of PBS buffer and then 200ul was loaded in a
Superose 12 column fitted to a computerised FPLC system from Pharmacia
(Sweden). The column was eluted as for the example above except that the
10 sensitivity of the UV detector was set at 1.0 absorbance units. The unreacted
lysozyme eluted at circa 19.65ml and the PEGylated lysozyme conjugates are
eluted at circa 10.49, 12.70, 14.32, 15.17 (Figure 2b). Again, the chromatogram
indicates that significant reaction occurs between the MPEG-Cl and lysozyme
under the above conditions.

15

**Comparative example 2: PEGylation of lysozyme with tresyl monomethoxy
PEG as the activated polymer**

68.3 mg of tresylated MPEG (TMPEG), prepared as previously
20 described [WO 95/06058], was reacted with 0.580 mg of lysozyme in a total
volume of 0.580 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23°C.
An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and
200ul was loaded, within a further 1 minute, onto a Superose 12 column fitted to
a computerised FPLC system from Pharmacia (Sweden). The column is eluted
25 with 25 ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring
(214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2
absorbance units. The unreacted lysozyme eluted at 19.40 ml and the PEGylated
lysozyme conjugates are eluted at circa 12.05, 14.24 and 15.74 ml (Figure 3).
From the profile it is evident that the PEGylation reaction is occurring at a

similar rate to that achieved with the MPEG-Cl sample in Example 2, which is surprising, given the anticipated low reactivity of the PEG-Cl.

Example 3 PEGylation of lysozyme at alkaline pH.

5

One disadvantage of the TMPEG method overcome by the present invention is that with the former, if the activated PEG is exposed to high pH, fluoride elimination occurs. This has two consequences: first the activated polymer is rapidly exhausted and, second, a proportion of the linkages made
10 between the polymer and the target molecule will have an alternate linkage (a sulphonate amide linkage as opposed to a secondary amine linkage). This linkage alters surface charge; introduces a coupling moiety into the product and the coupling between the polymer and target molecule or structure is unstable, particularly at alkaline pH. MPEG-Cl does not break down as rapidly as TMPEG
15 at alkaline pH (see Example 9 below).

50.0 mg of activated MPEG-Cl was reacted with 0.455 ml of 1mg/ml lysozyme (Fluka) in phosphate buffer (pH 8.66) for 21 minutes at 26°C. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was
20 loaded in a Superose 12 column and eluted as in Example 2. The chromatogram shows that one peak elutes at circa 17.03 ml and other peaks eluted at circa 12.64, 14.32 (largest) (Figure 4). There was no peak at the location of unmodified lysozyme (circa 19.2ml) indicating that the MPEG-Cl and lysozyme react significantly faster under the above conditions. It should be noted that there
25 is no basis for the formation of an alternate linkage with the MPEG-Cl method.

Example 4: PEGylation of lysozyme at 4°C

One further disadvantage of the TMPEG method overcome by the present invention is that, with the former, if the activated PEG is used at low temperature, longer PEGylation times and/or higher polymer concentrations are required to achieve the same degree of PEGylation as achieved at room temperature. With TMPEG, however, the duration of the PEGylation reaction cannot be much prolonged since the activated polymer hydrolyses at a significant rate (see Example 9 below). The ability to PEGylate substrates at low temperatures can be of advantage with target molecules or structures that are unstable at higher temperatures. In addition, polymer concentrations can be lowered significantly if longer reaction times are feasible.

49.80mg of MPEG-Cl was reacted with 0.453mL of 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at 4°C. The initial polymer concentration was 110 mg/mL. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded on a Superose 12 column and eluted as in Example 2. The unreacted lysozyme eluted at circa 19.55ml and the PEGylated lysozyme conjugates eluted at circa 10.61, 12.61, 14.28, 15.10 (Figure 5a). The chromatogram indicates that although some reaction has occurred between the MPEG-Cl and the lysozyme the proportion of unmodified material is higher than was observed with similar reactions carried out at room temperature (see Figures 2a and 2b).

However, this reaction could be prolonged until essentially all lysozyme had reacted. 47.89mg of MPEG-Cl was reacted with 0.435mL of 1mg/ml lysozyme (Fluka) in phosphate buffer for 72.5 hours at 4°C. As above, The initial lysozyme concentration was 110 mg/ml. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded in a Superose 12 column and eluted as in Example 2. The unreacted lysozyme was indistinguishable among three fragments at 16.91, 18.02 and 19.31. The

PEGylated lysozyme conjugates eluted at circa 12.72 and 14.86 (Figure 5b). The chromatogram indicates that almost complete reaction has occurred between MPEG-Cl and lysozyme.

5 **Comparative example 4: PEGylation using TMPEG at reduced temperature.**

Figure 6 shows the result of reaction of lysozyme with TMPEG at three different reaction temperatures.

10

135mg of TMPEG-12K was reacted with 0.5ml of 1mg/ml lysozyme (Fluka) in phosphate buffer for 21minutes. The reaction was performed three times at different temperatures: 3°C, 15.5°C and 22.5°C. 100ml of each reaction mixture was diluted with 400ul of PBS buffer and then 200ul was
15 loaded on a Superose 12 column and eluted as in Example 2.

The estimated areas under the curve for the unmodified lysozyme peak were 52.5% at 3°C, 46.5% at 15.5°C and 23% at 22.5°C.

20 **Example 5: An additional synthetic route for MPEG-Cl**

MPEG-Cl is also produced by variation of the previously reported manufacturing procedure for TMPEG [WO 95/06058]. This product is exposed to more rigorous washing steps than MPEG-Cl derived from the
25 thionyl chloride method and is included here because this may be the basis of the observed superior retention of bioactivity.

MPEG (Mr 5000; 18g; Shearwater Polymers Inc, USA) was dissolved in toluene (40ml) and the water-organic azeotrope was distilled off, followed

by the bulk of the toluene (109-110°C), obtaining about 35 ml of distillate. The remaining toluene was removed by rotary evaporation under reduced pressure.

5 The dried MPEG was dissolved in a dry acetonitrile (40ml; dried overnight with molecular sieve 3A (3Angstrom), BDH, UK, added at 10g per 50ml) at room temperature and then cooled in a water-ice bath to 1°C and magnetically stirred. One ml of ice-cold pyridine (BDH, UK) was added over
10 1 min with constant stirring. Tresyl chloride (1ml; Fluka AG, Switzerland) was then added drop-wise to the stirred solution over 5 min. The solution was then placed at room temperature and stirring was continued for a further 2 h. Acetonitrile was then removed under reduced pressure with occasional warming with a 70°C water bath.

15 The solid product was dissolved in methanol-HCl (300ml; prepared using 0.75ml conc HCl to 2.5l methanol) and cooled to -20°C overnight. The white precipitate was collected by centrifugation at 0°C and redissolved in 200ml of methanol-HCl. The solution was cooled in ice/salt for 30min and the precipitate isolated by centrifugation. To free the sample of pyridine, the
20 process was repeated until the absorbance of the supernatant at 255nm was at a minimum. Typically 12 washes are required and the minimum absorbance (1cm path length) is 0.02, in this instance, the minimum was 0.04 and 14 washes were used without further improvement. The sample was then dissolved in methanol (200ml) and reprecipitated twice before being dried by
25 rotary evaporation, and then overnight in a freeze dryer (yield 16g).

Analysis by reverse phase liquid chromatography was performed as in Example 1 using a Polymer Laboratories PLRP-6 column and a PL- EMD960 mass detector (Figure 7). The product contained negligible amounts of MPEG

($<1\%$, see peak at 15.0min) and of the activated PEG species 92.6% was MPEG-Cl (peak at 17.7min) and 7.4% was TMPEG (peak at 19.3min). ^1H -nmr and ^{19}F -nmr showed the sample to be substantially MPEG-Cl with some TMPEG. Elemental analysis detected chlorine 0.58% (theoretical chlorine content for 100% MPEG-Cl of molecular weight 5K is 0.7%)

Example 5A Preparation of PEG protein conjugates

70.4 mg of MPEG-Cl produced by the method of Example 5 were reacted with 0.640 mg of lysozyme in a total volume of 0.640 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23°C . An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 3 minutes, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 25 ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The unreacted lysozyme is eluted at circa 19.28 ml and the PEGylated lysozyme conjugates eluted at circa 12.38, 14.38, 14.88 and 15.64 ml (Figure8).

20

Thus reaction with lysozyme (Figure 8) showed only a slightly lower reactivity than the MPEG-Cl prepared as in Example 1 and reacted with lysozyme in Figures 2a and b. That the reactivity was due to the MPEG-Cl and not the contaminating TMPEG was demonstrated in two ways: first by showing that an equivalent amount of TMPEG to that contaminating the MPEG-Cl preparation had a much lower reactivity (comparative Example 5 Figure 9), and second, that after prolonged hydrolysis sufficient to convert the residual TMPEG to MPEG the remaining MPEG-Cl still reacted (Example 6, Figure 10).

25

Comparative example 5: PEGylation of lysozyme with tresyl monomethoxy PEG as the activated polymer

6.8 mg of tresylated MPEG (92.2% purity, with 8.8% MPEG-C1 and negligible MPEG; assessed by reverse phase chromatography using a Polymer Laboratories PLRP-6 column and a PL-EMD960 mass detector) was reacted with 0.580 mg of lysozyme in a total volume of 0.580 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23°C. An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 1 minute, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column is eluted with 25ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The amount of tresylated MPEG used in this reaction was reduced ten fold relative to the MPEG-C1 preparation used in example 5 (i.e. to an amount slightly more than the contaminating TMPEG present in Example 5), however much less PEGylation was observed (Figure 9) than that observed in the MPEG-C1 example (Figure 8), despite the TMPEG exposure being similar. Thus showing that the reaction in Example 5 was due to the MPEG-C1.

Example 6: Preparation of PEG-protein conjugates after hydrolysis of residual TMPEG in the MPEG-C1 sample of Example 5.

An aliquot of the MPEG-C1 sample (105mg) prepared in Example 5 was subjected to hydrolysis in water (525ul) for 17 days. After this the sample contained 82.4% MPEG-C1, 0% TMPEG and 17.6% MPEG (assessed by reverse phase chromatography using a Polymer Laboratories PLRP-6 column and a PL-EMD960 mass detector).

275 ul of this sample was reacted with 0.5mg of lysozyme in a total volume of 0.5 ml of 20mM phosphate buffer, pH7 for 21 minutes at 23°C. An aliquot (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 1-2 minutes, onto a Superose 12 column
5 fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 25ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The unreacted lysozyme eluted at 18.75ml and the PEGylated lysozyme conjugates are eluted as a shouldered peak at circa
10 14.33ml with two subsidiary shoulders evident at slightly higher elution volumes (Figure 10). From the profile it is evident that the PEGylation reaction is surprisingly, given the theoretically anticipated very low reactivity of MPEG-Cl, still occurring at a significant rate despite the absence of any TMPEG and despite conversion of a proportion of the MPEG-Cl to MPEG.

15

Example 7: PEGylation of GM-CSF and retention of bioactivity

10 ul of GM-CSF (Hoescht) at 10 ug/ml in PBS were mixed with 15 ul of a solution of MPEG-Cl (produced by the method of Example 5) at circa 250
20 mg/ml and 15 ul of sterile PBS (Gibco) in a sterile eppendorf tube. Sham treatment controls were set up with MPEG-5K obtained from Union Carbide. The reaction mixture was incubated using a rotary mixer for 2 h at room temperature. It has previously been established that, given the reactivity rate of the activated polymer, these reaction conditions produce a statistical mixture
25 of PEGylated GM-CSF products with mainly 1-3 PEG chains per molecule and over 75% modification. 8 ul of reaction mixture were then added to 10 ml of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum, Life Technologies) to obtain a solution of GM-CSF at 2 ng/ml. The bioactivity of the samples was tested in thymidine uptake assays using a GM-

CSF responsive cell line in 96 well microtiter plates (Nunc). The samples were diluted with fully supplemented RPMI-1640 corrected for PBS content (64 ul of sterile PBS were added to 80 ml of RPMI-1640) in order to obtain a range of concentrations of GM-CSF from 0.05 to 0.5 ng/ml. The 150 ul solution of GM-CSF in each well received 5×10^5 TF-1 cells (starved for 24h, i.e. grown for 24h without addition of GM-CSF) and the plate was then incubated for 18h at 37°C under 5% CO₂ atmosphere. The growth stimulation is then quantified using ³H-Thymidine incorporation. [³H]-Thymidine stock (Amersham - TRK120 - batch: B395) was 100 fold diluted and 50 ul of this solution were added to each well. The plate was further incubated for 4h at 37°C under 5% CO₂ atmosphere. The cells were harvested onto a glass filter (Wallac, size 90x120mm), the filter was dried for 2h at 75°C and the dried filter was transferred to a bag (Wallac, size 90x120mm) containing 5 ml of scintillation liquid (Wallac, Betaplate Scint.). the beta emission was quantified using a beta counter (Wallac, 1450 Microbeta plus). The data were background subtracted and CPM-background was plotted against GM-CSF concentration (ng/ml). This experiment was repeated 3 times (Figure 11a-c). The incubation of GM-CSF with this batch of MPEG-Cl resulted in a conservation of bioactivity of 52.8% +/- 5.8%.

Example 8 : PEGylation of Erythropoietin (EPO) and retention of bioactivity.

5 ul of EPO (Cilag) at 3200 U/ml in PBS were mixed with 29 ul of an MPEG-Cl solution at circa 250 mg/ml and 61 ul of sterile PBS (Gibco) in a sterile eppendorf tube. The MPEG-Cl was produced by the method of Example 1. Control sham-treated samples were also prepared using MPEG (obtained from Shearwater Polymers Inc.). The reaction mixture was incubated

using a rotary mixer for 2 h at room temperature. 705 ul of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum) (Life Technologies) were then added to 95 ul of reaction mixture to obtain a solution of EPO at 20 U/ml. The bioactivity of the samples was tested in a 96 wells microtiter plate (Nunc). The samples were diluted with fully supplemented RPMI-1640 corrected for PBS (9.5 ml of sterile PBS were added to 70.5 ml of RPMI-1640) in order to obtain a range of EPO concentrations from 1 to 10 U/ml. The 150 ul solution of EPO in each well received 5×10^5 TF-1 cells (starved for 24h, i.e. grown for 24h without addition of GM-CSF, the usual cell growth support for this cell line) and the plate was incubated for 18h at 37°C under 5% CO₂ atmosphere. The growth stimulation was then quantified using [³H]-Thymidine incorporation. [³H]-Thymidine stock (Amersham - TRK120 - batch: B395) was 100 fold diluted and 50 ul of this solution were added to each well. The plate was further incubated for 4h at 37°C under 5% CO₂ atmosphere. The cells were harvested onto a glass filter (Wallac, size 90x120mm), the filter was dried for 2h at 75°C and the dried filter was transferred to a bag (Wallac, size 90x120mm) containing 5 ml of scintillation liquid (Wallac, Betaplate Scint.). the beta emission was quantified using a beta counter (Wallac, 1450 Microbeta plus).

20

The data were background subtracted and CPM-background were plotted against EPO concentration (as U/ml, i.e. without adjusting for loss of native activity; Figure 12). There was no significant loss of bioactivity.

25

5 ul of EPO (Cilag) at 3200 U/ml in PBS were mixed with 29 ul of an MPEG-Cl solution at circa 250 mg/ml and 61 ul of sterile PBS (Gibco) in a sterile eppendorf tube. The MPEG-Cl was prepared as in Example 5. Sham treated control EPO was also prepared using MPEG-5K obtained from Union Carbide. The reaction mixture incubated using a rotary mixer for 2 h at room

temperature. 705 ul of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum, Life Technologies) were then added to 95 ul of reaction mixture to obtain a solution of EPO at 20 U/ml. The bioactivity of the samples was tested as described for Figure 12.

5

This experiment was repeated 3 times. The data were background subtracted and CPM-Background were plotted against EPO concentration (U/ml) (Figure 13a-c). At low doses of the reaction products, the dose response curves were superimposable, but at higher doses in two experiments there was progressive departure between the test and control curves. This indicates the presence of some toxic or inhibitory material (the EPO assay is particularly sensitive to inhibition, much more so than the GM-CSF assay). The level of toxicity observed is lower than for several other PEGylation procedures previously examined (cf. the cyanuric chloride method and the phenylchloroformate method (*Francis, G.E. et al (1996) J. Drug Targeting* 3 321-340)). The superimposition of the upward part of the test and control dose response curves at low doses of test material indicates no significant loss of bioactivity.

20

Example 9 and comparative Example 9: Breakdown of MPEG-Cl and TMPEG in aqueous solution.

One advantage of the present invention is the relative stability of the activated polymer.

25

Figure 14 shows the effect for MPEG-Cl and Figure 15 a-c shows the breakdown of three TMPEG samples.

Figures 14 and 15a-c compare the breakdown rates at pH 7 for MPEG-Cl made by the method of example 1 and three TMPEG samples made by different manufacturing techniques.

5 The release of acid and of fluoride from samples of activated MPEGs at pH 7 and also at pH 9 were measured in a pH-stat (Mettler Toledo DL 77 titrator) fitted with a fluoride electrode (Mettler Toledo DX 219) and pH electrode (Metler Toledo DG101-SC). 25 ml of 0.9% NaCl was adjusted to pH 7 or 9 with approximately 0.01 M NaOH (standardised by potassium
10 hydrogen phthalate titration). MPEG-Cl or TMPEG (approx 100mg; approx 20 umoles) was added to the saline and simultaneous measurements of fluoride concentration and alkali consumed were made at 20 second intervals for up to 60 min. Results are plotted as umoles of fluoride present and acid produced as a function of time. Samples of activated polymers
15 dissolved in the NaCl required NaOH to bring to the starting pH, indicating that they were not neutral but were somewhat acidic. This immediate uptake of alkali was then followed by a steady uptake as acid was released progressively, and is seen as an intercept at zero time in the plot of alkali uptake against time. This value can be subtracted to provide a plot of acid
20 release at the selected pH.

The changes in fluoride in Figure 14 are an artefact of the impact of OH ions on the fluoride electrode.

25 Figure 15a shows a sample of TMPEG prepared as previously described [WO 95/06058] with very low fluoride elimination. Note that its acid elimination is substantially higher than that of the MPEG-Cl sample. Figures 15b and c show two samples of TMPEG (obtained from Sigma and Shearwater Polymers Inc) made by different manufacturing procedures and

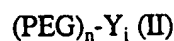
exhibiting higher fluoride elimination. A combination of hydrolysis to MPEG and trifluoroethanesulphonic acid and fluoride elimination thus produces substantially faster breakdown of TMPEG versus MPEG-Cl. Note that MPEG-Cl left in water for 17 days still retained good reactivity (see above), implying
5 that the acid elimination rates at acidic pH is even lower than that at pH 7.

Figures 16 and 17 show comparable break down rates for MPEG-Cl and TMPEG at pH 9.0.

CLAIMS

1. A process for the PEGylation of a substrate, comprising the reaction of
5 a halogenated PEG with the substrate and the PEG is bound directly to
the substrate, with the proviso that the substrate is not a steroid or
when the halogenated PEG is PEG-bromide the substrate is not 5-
fluorouracil.

10 2. A process according to claim 1 wherein the halogenated PEG is of
general formula I or II



wherein:

15

PEG is a bivalent group of formula $-(\text{-CH}_2\text{CH}_2\text{O-})_m\text{-CH}_2\text{CH}_2\text{-}$,

where m is equal or greater than 1, derived from a polyethylene
glycol; X is a halogen atom, a blocking group or an activating group
capable of coupling the PEG moiety to another moiety; Y is a halogen;

20

n is the number of PEG termini; n is equal or greater than 2; i is
equal or less than n and i/n PEG termini are substituted by Y in
compounds of Formula II.

3. A process according to claim 2 wherein Y is Cl, Br or I.

25

4. A process according to either of claims 2 and 3 wherein X is a
blocking group and is methyl, t-butyl or benzyl ether.

5. A process according to either of claims 2 and 3, wherein X is a tresyl activating group.
6. A process according either of claims 1 and 2, wherein the halogenated PEG is one of; methoxy-PEG-Cl, methoxy-PEG-Br, methoxy-PEG-I, Cl-PEG-Cl, Br-PEG-Br or I-PEG-I.
7. A process according to either of claims 1 and 2 wherein the substrate is selected from proteins, peptides, DNA, RNA, nucleotides, nucleotide analogues, hormones other than steroids, antibiotics, liposomes, viruses, unicellular organisms, micelles, metallic plastic, or polymeric surfaces.
8. A product produced by a process according to any of claims 1 to 7.
9. A product according to claim 8, for use in medical therapy.
10. A pharmaceutical formulation comprising a product according to claim 8.
11. The use of a product according to claim 9 or a formulation according to claim 10, in the manufacture of a medicament for use in medical therapy.
12. A process according to claim 1, a product according to claim 8, a formulation according to claim 10 or a use according to claim 11, substantially as hereinbefore described with reference to the accompanying Figures and Examples.

13. A reagent comprising MPEG-halide obtainable by the reaction of MPEG with tresylhalide wherein at least a part of any TMPEG produced in the reaction is removed.

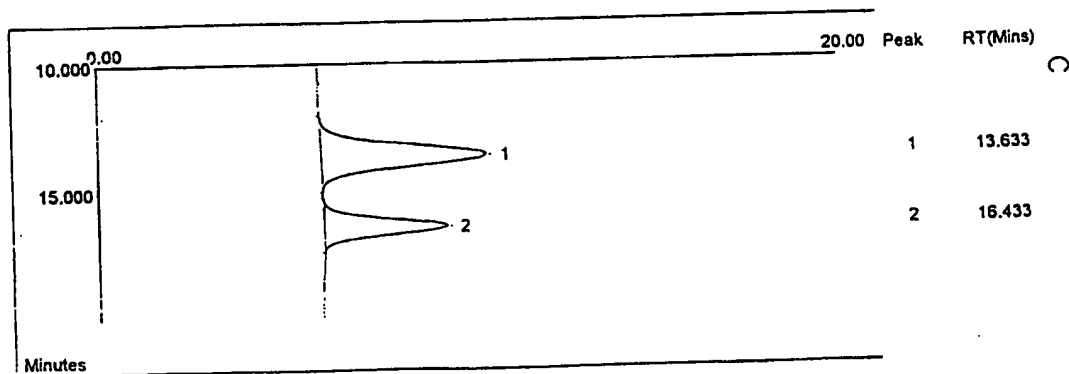
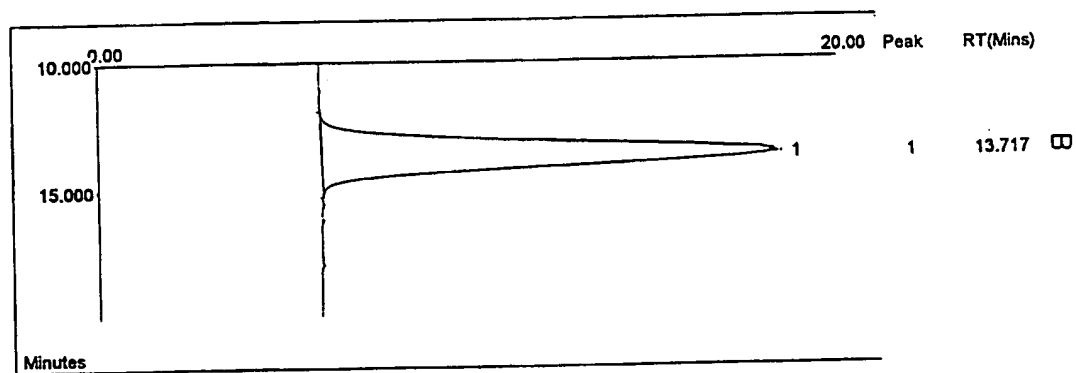
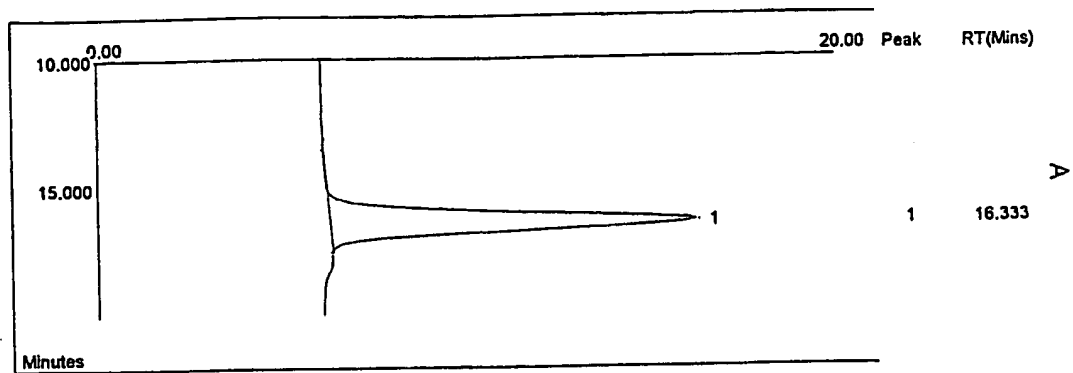


FIGURE 1

Figure 2

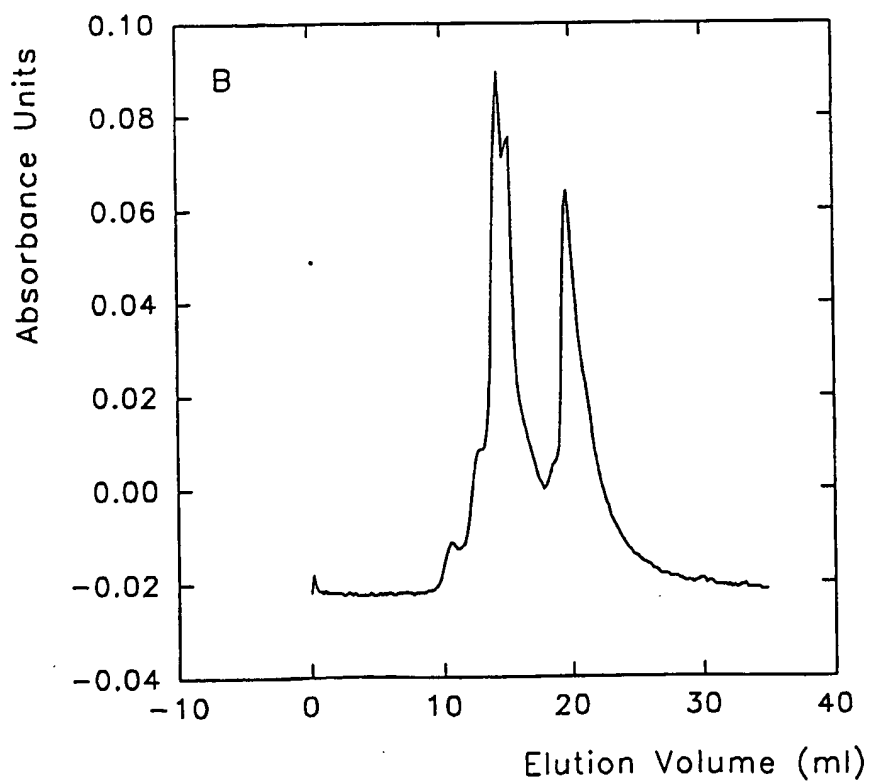
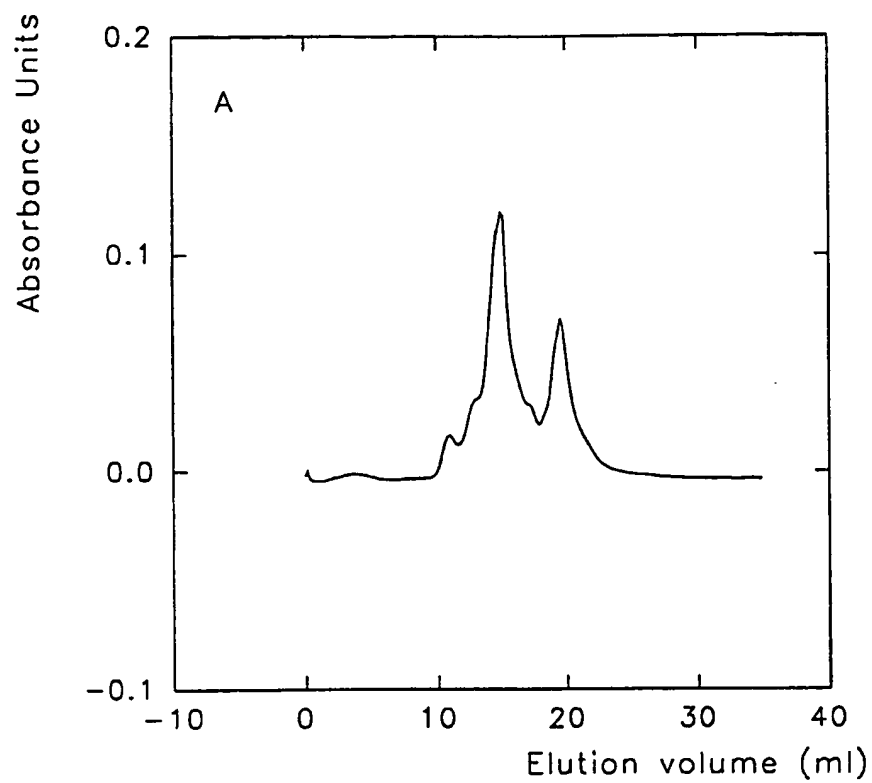


Figure 3

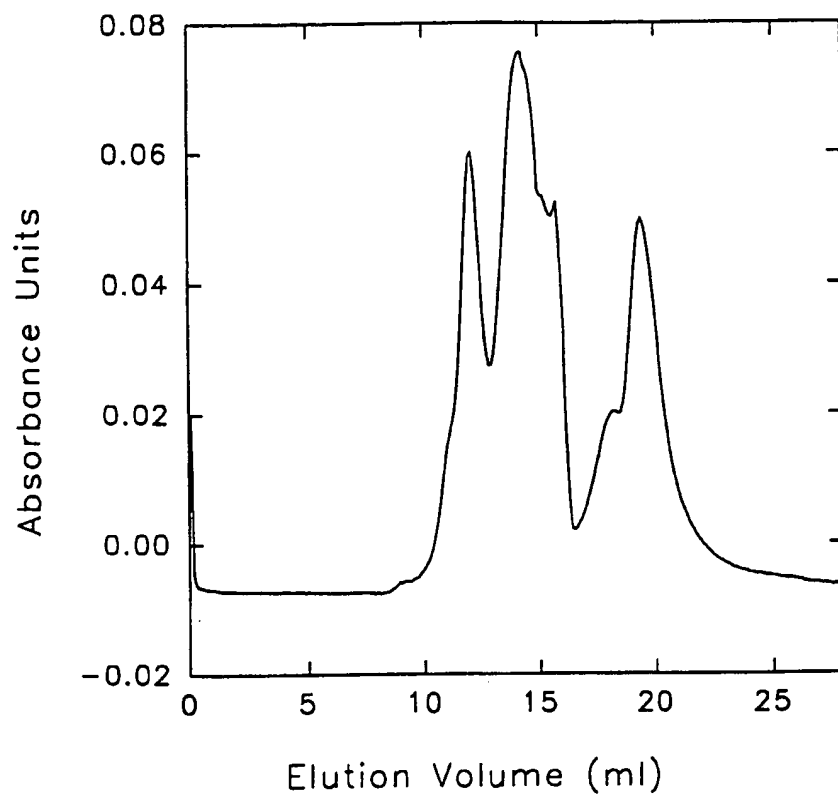


Figure 4

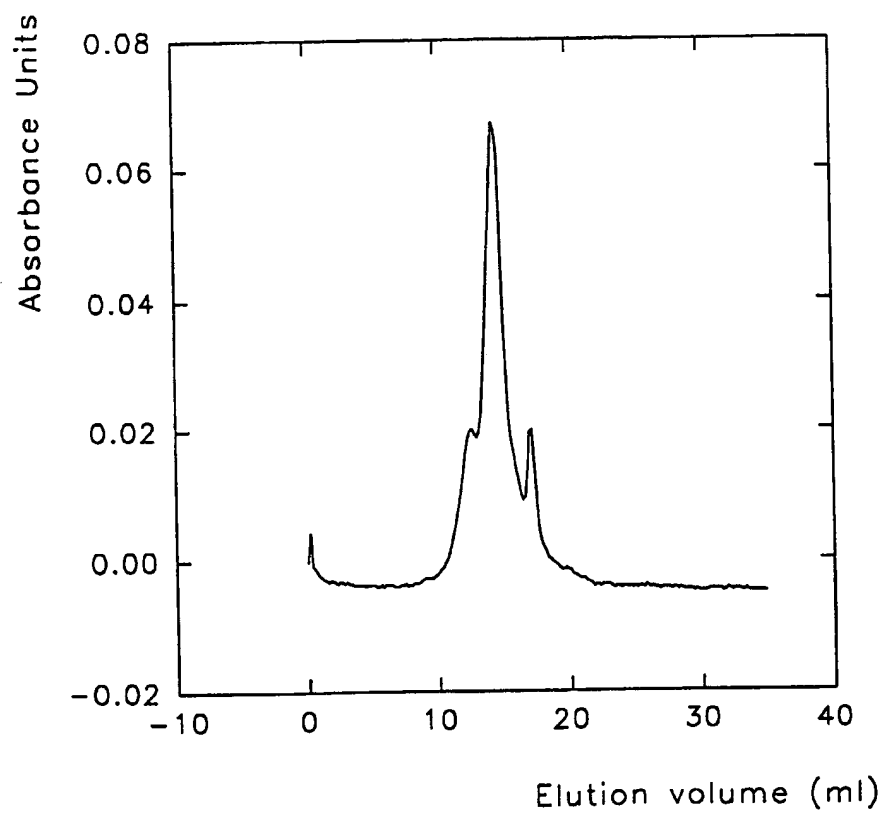


Figure 5

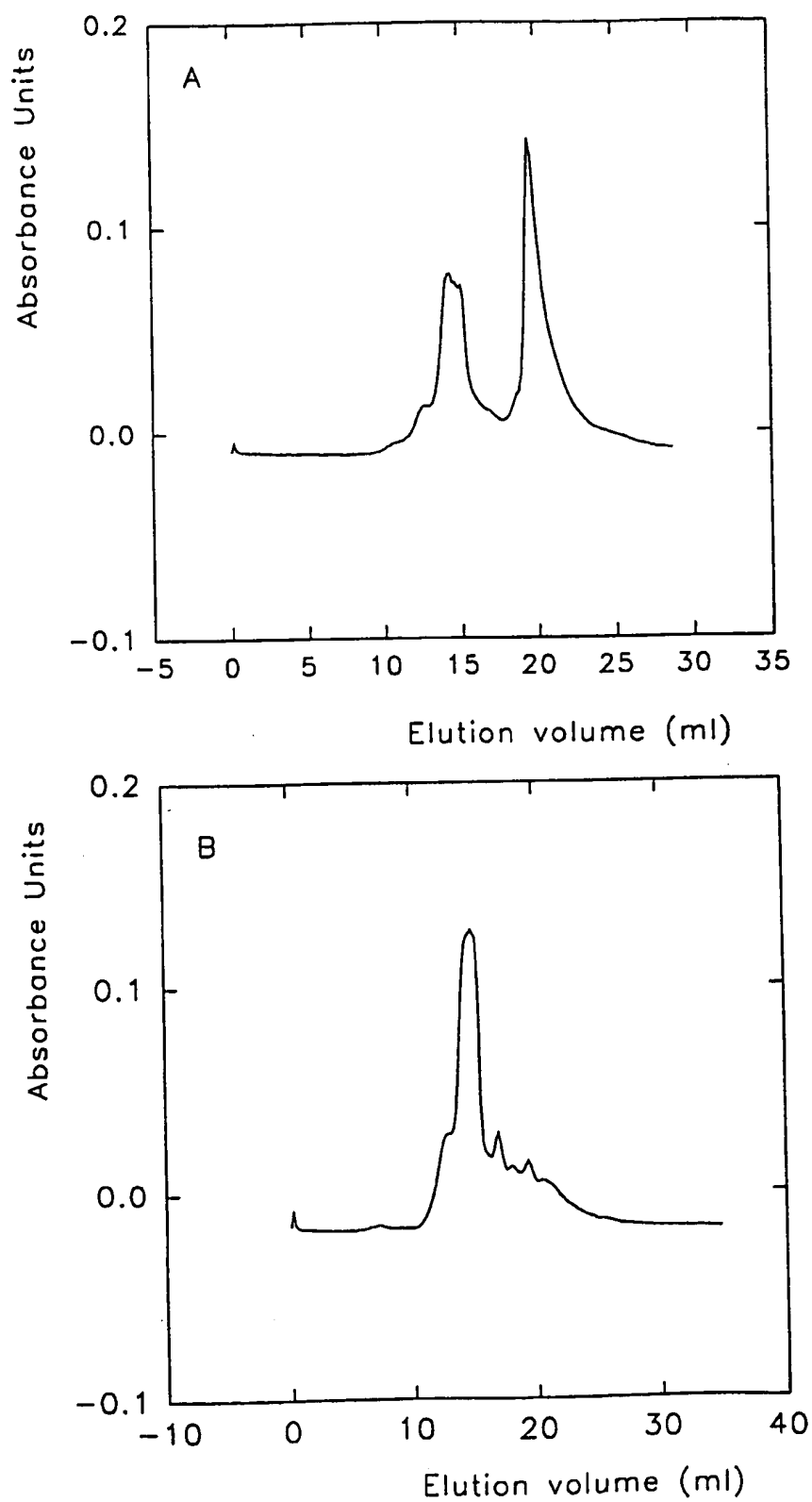


Figure 6

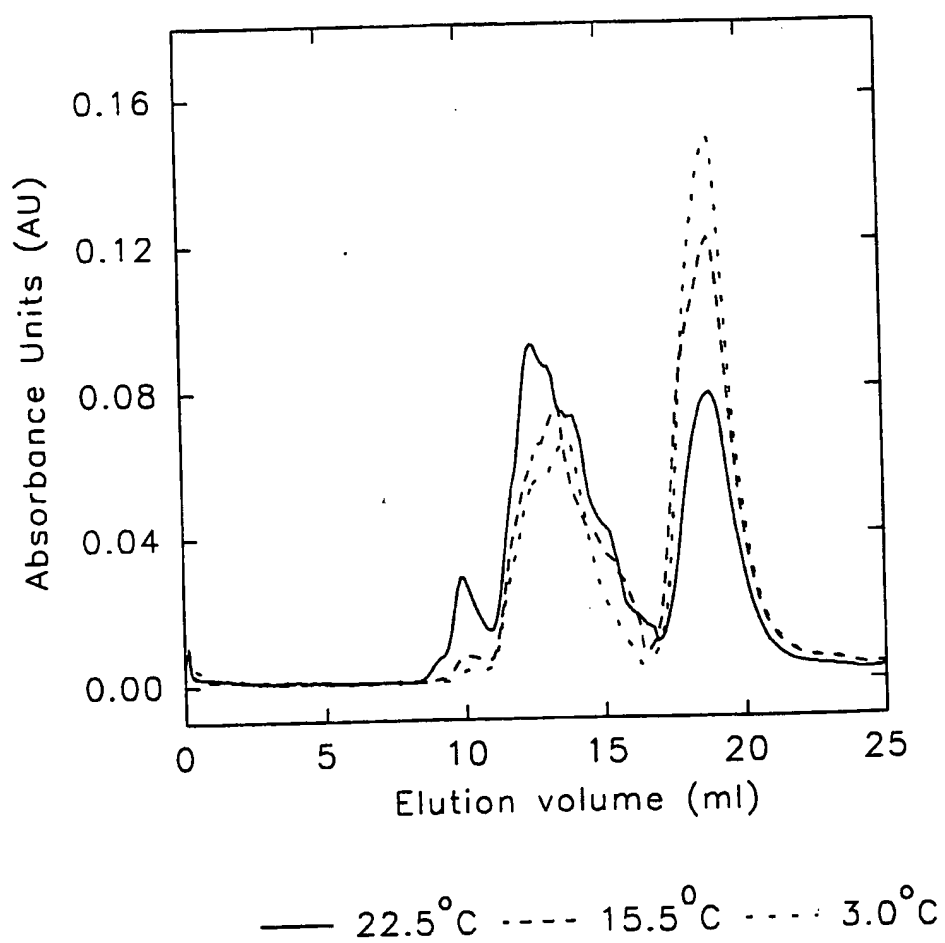


FIGURE 7

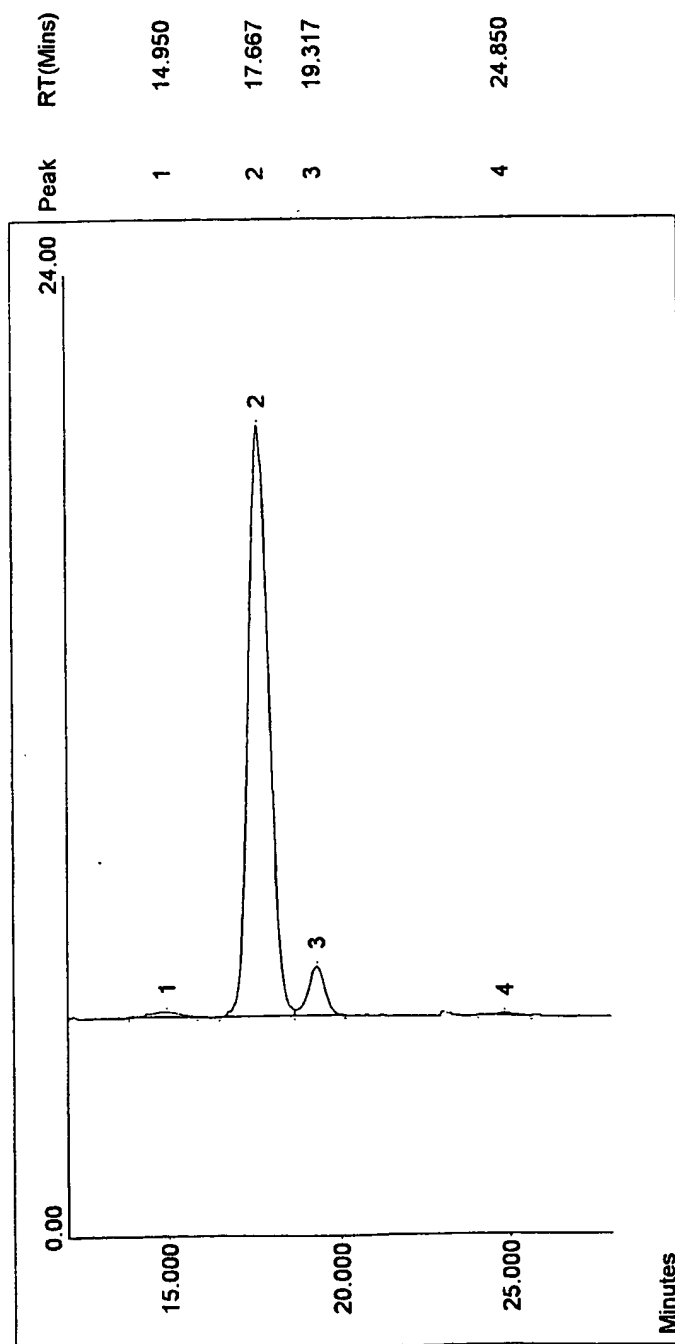


Figure 8

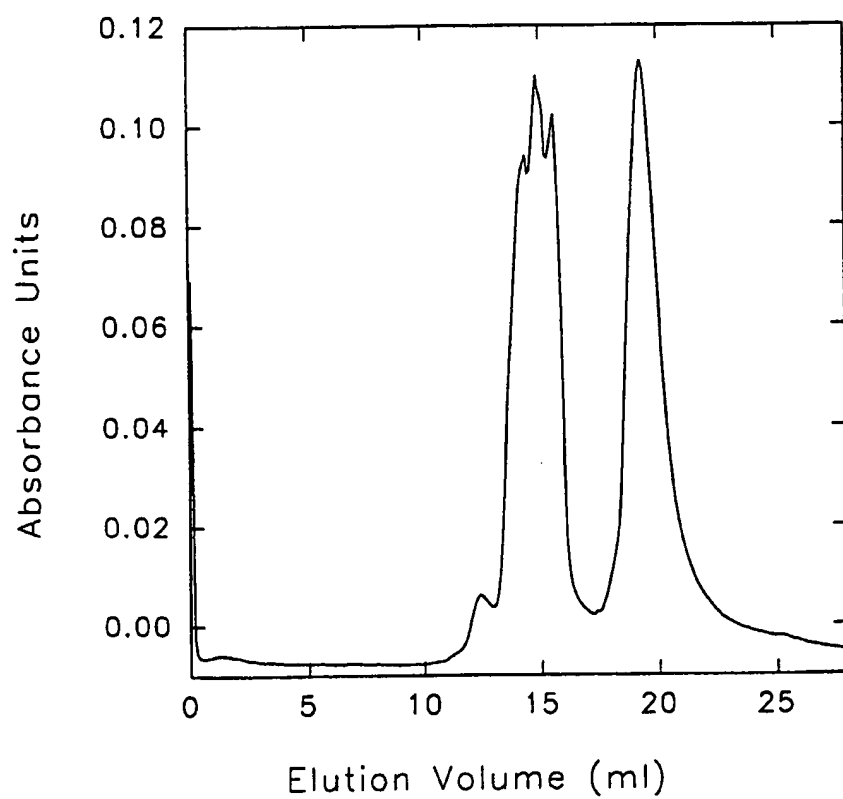


Figure 9

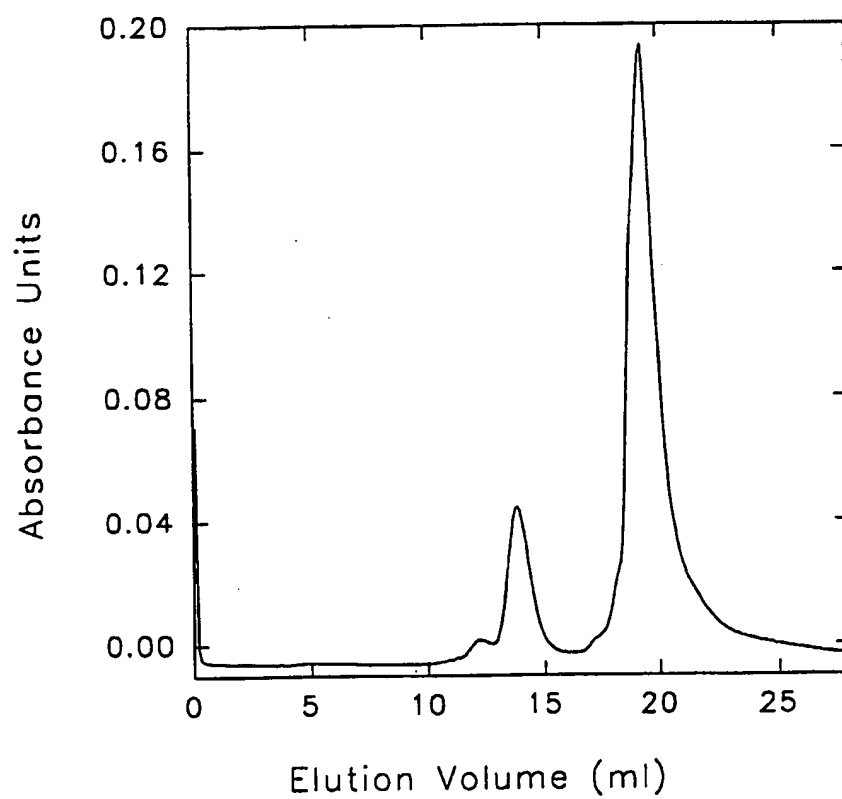


Figure 10

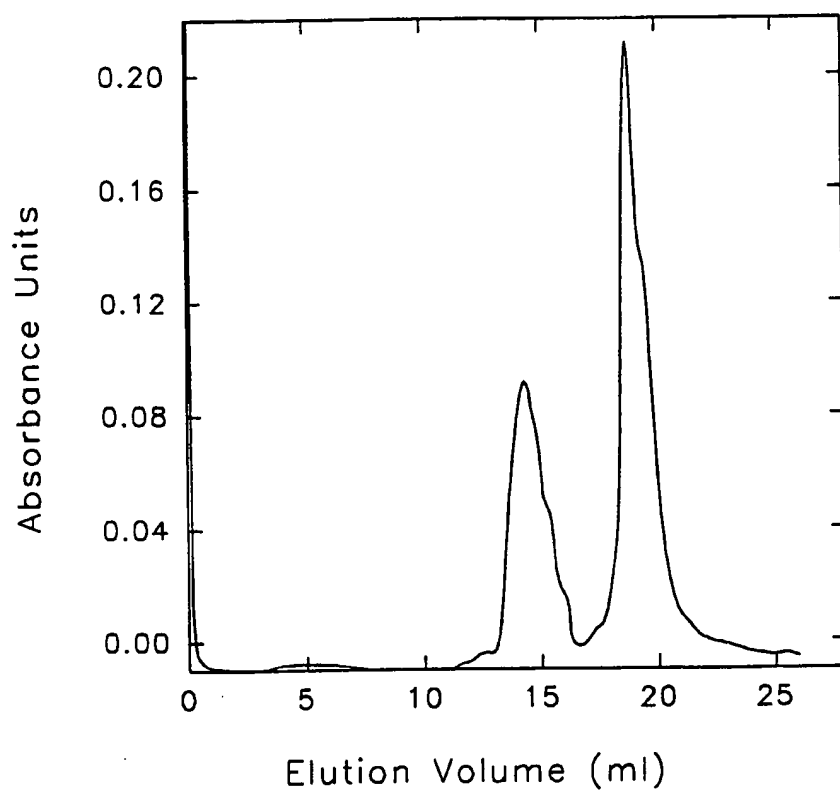


FIGURE 11

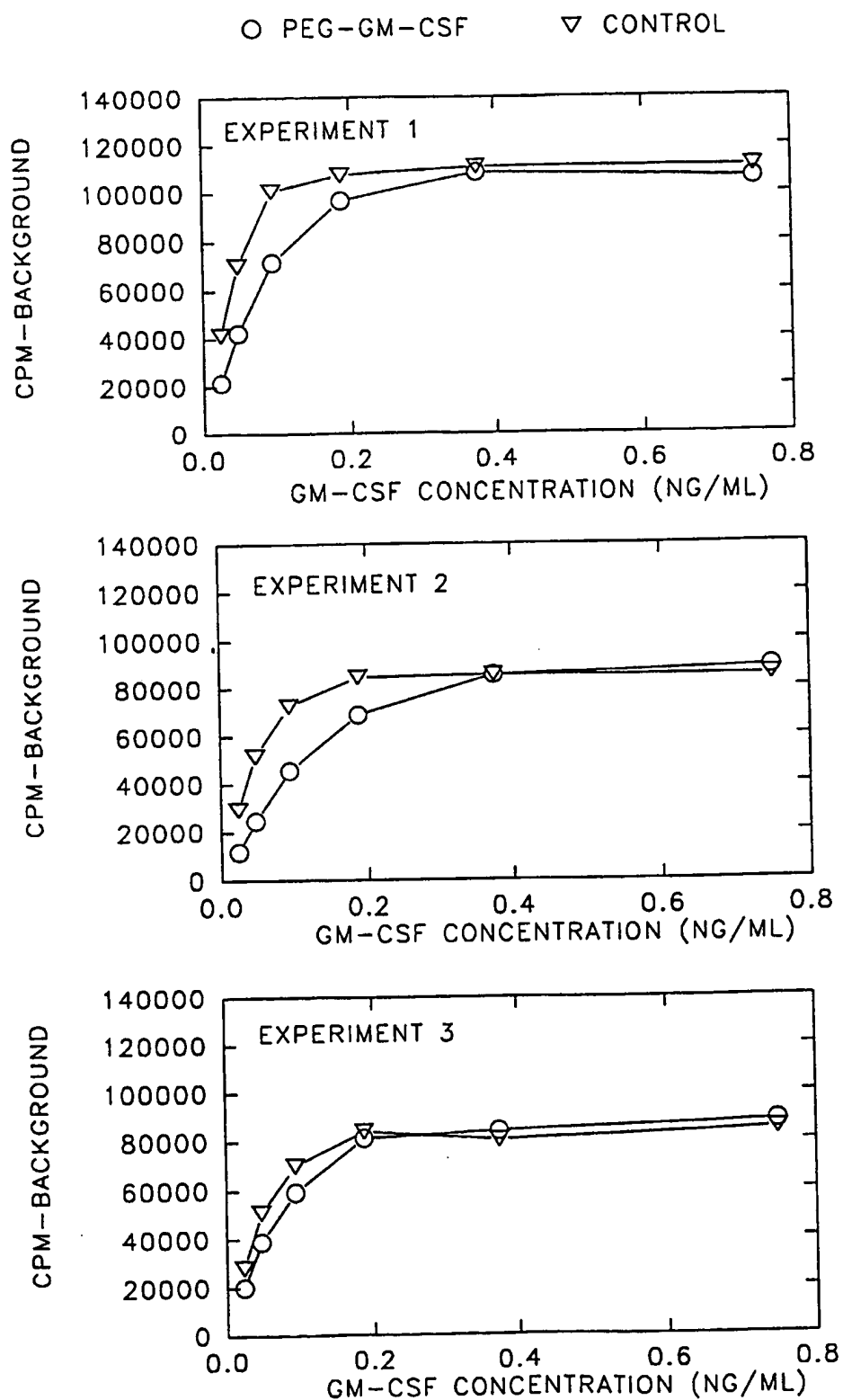


FIGURE 12

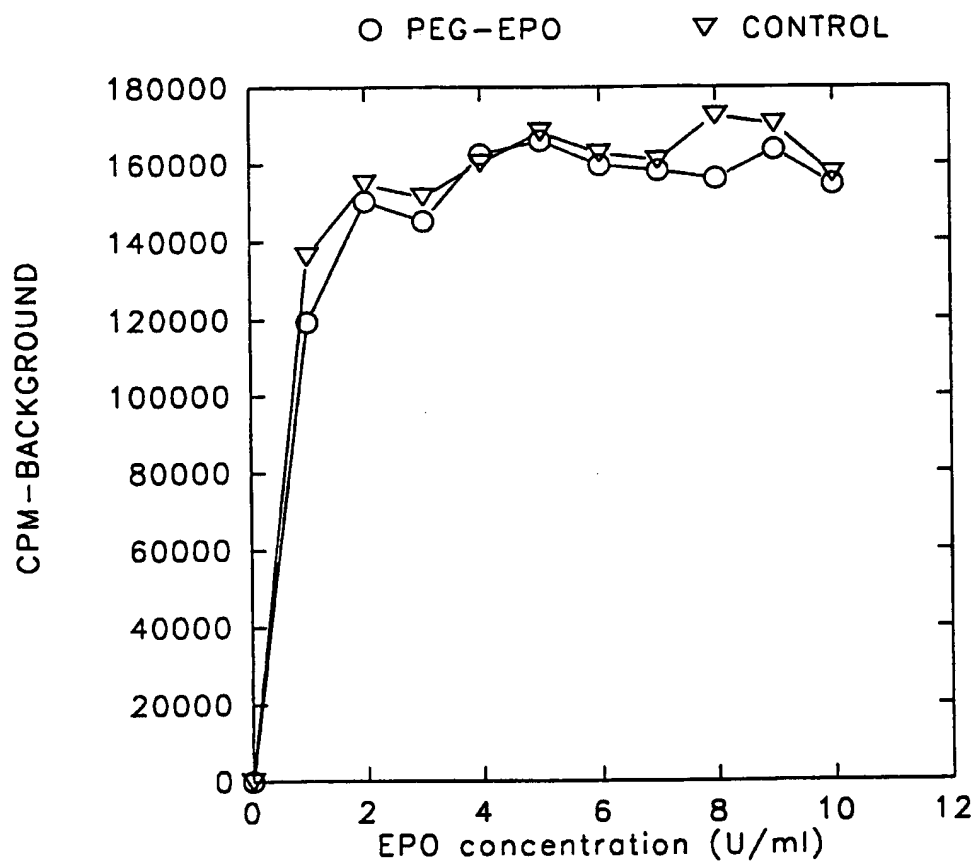


FIGURE 13

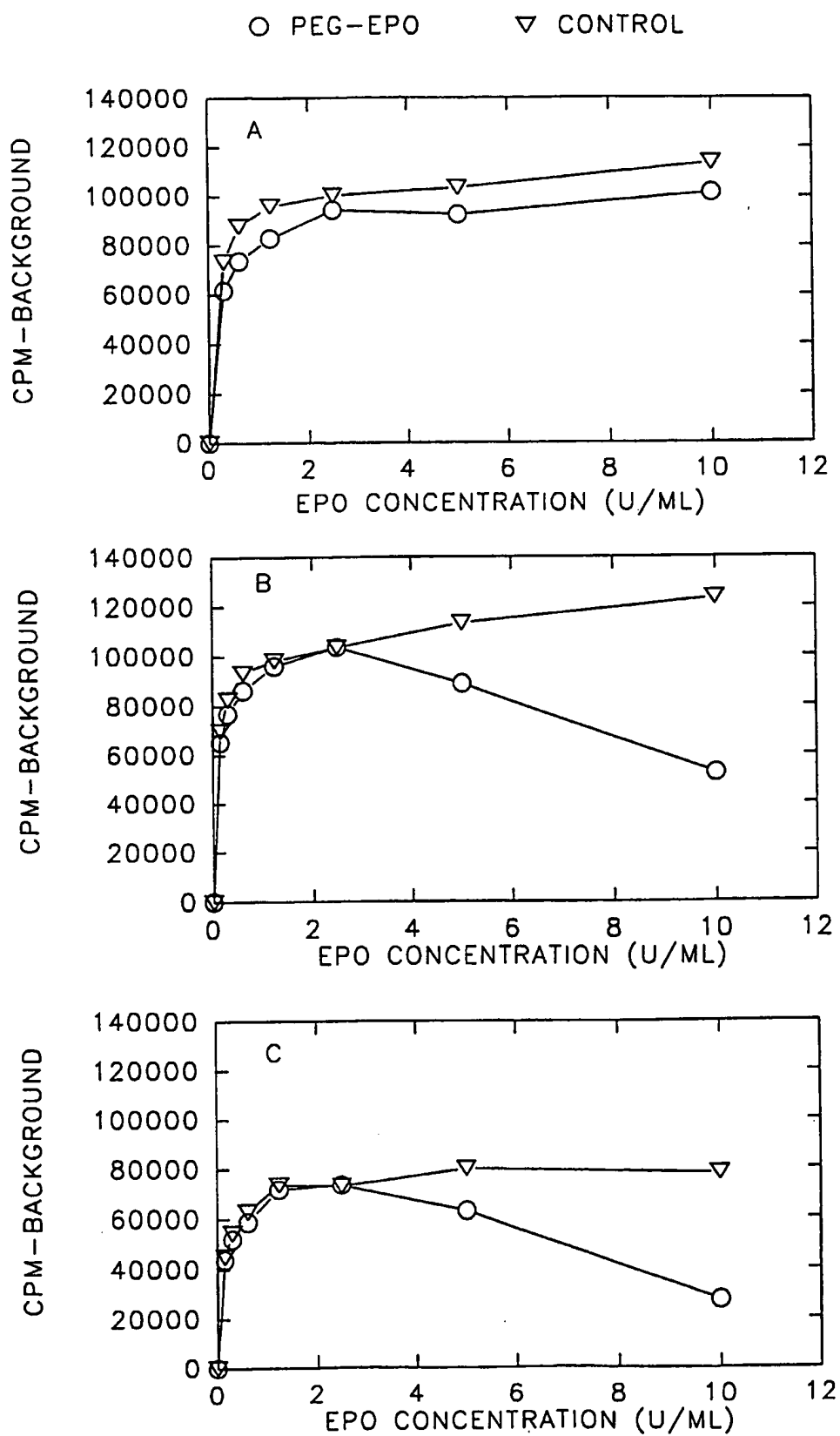


Figure 14

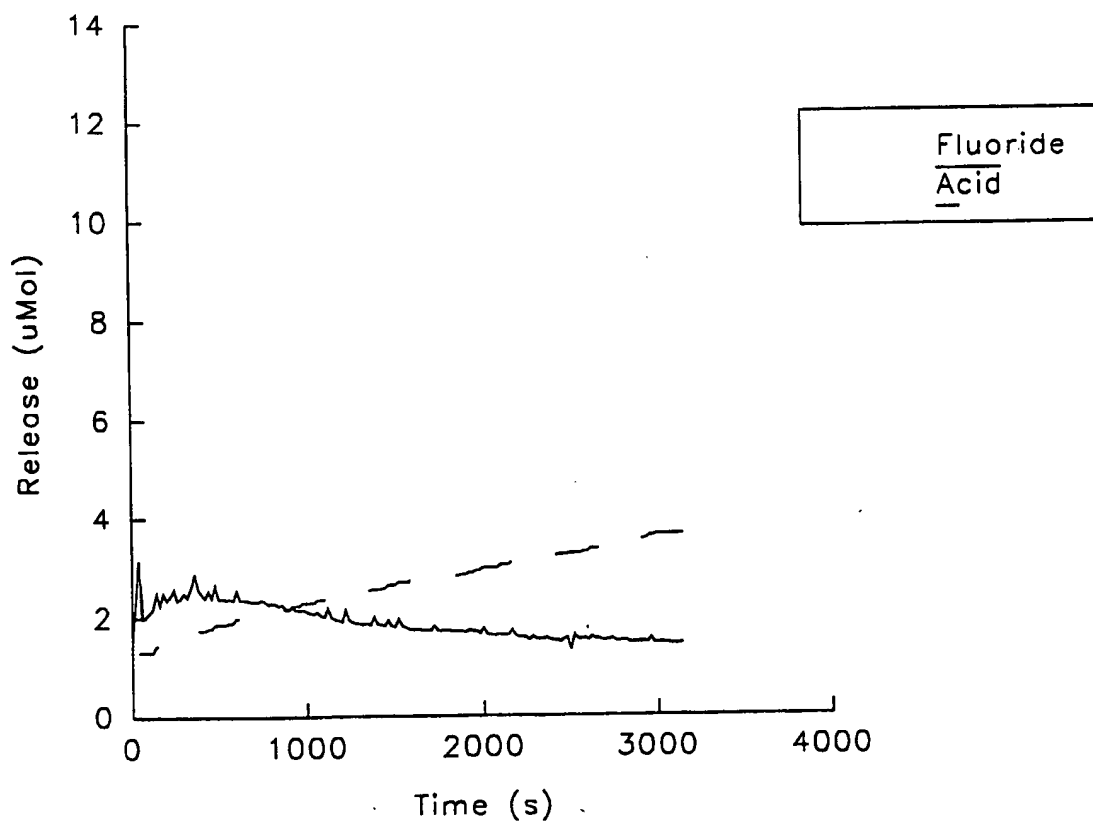


Figure 15

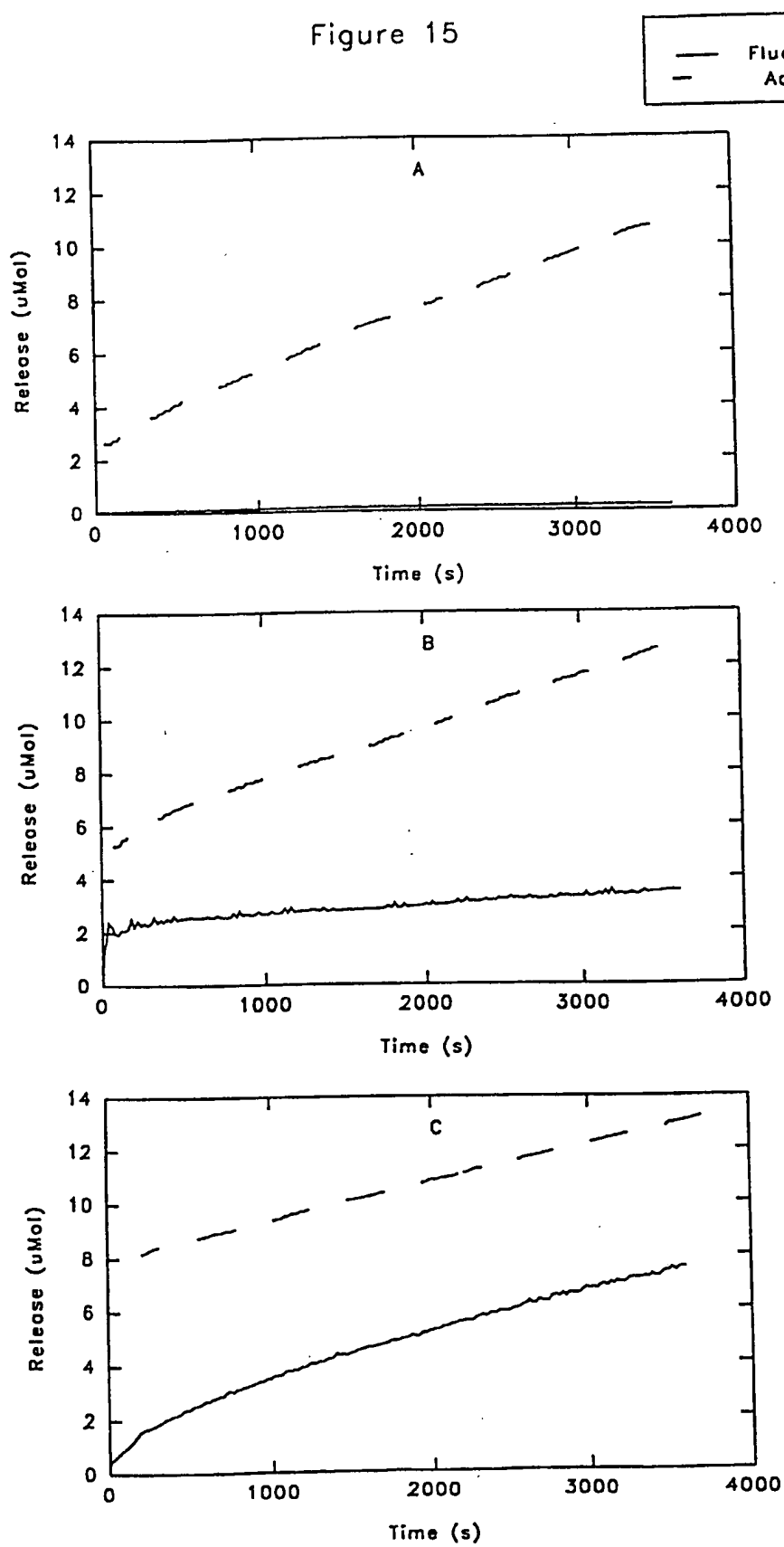


Figure 16

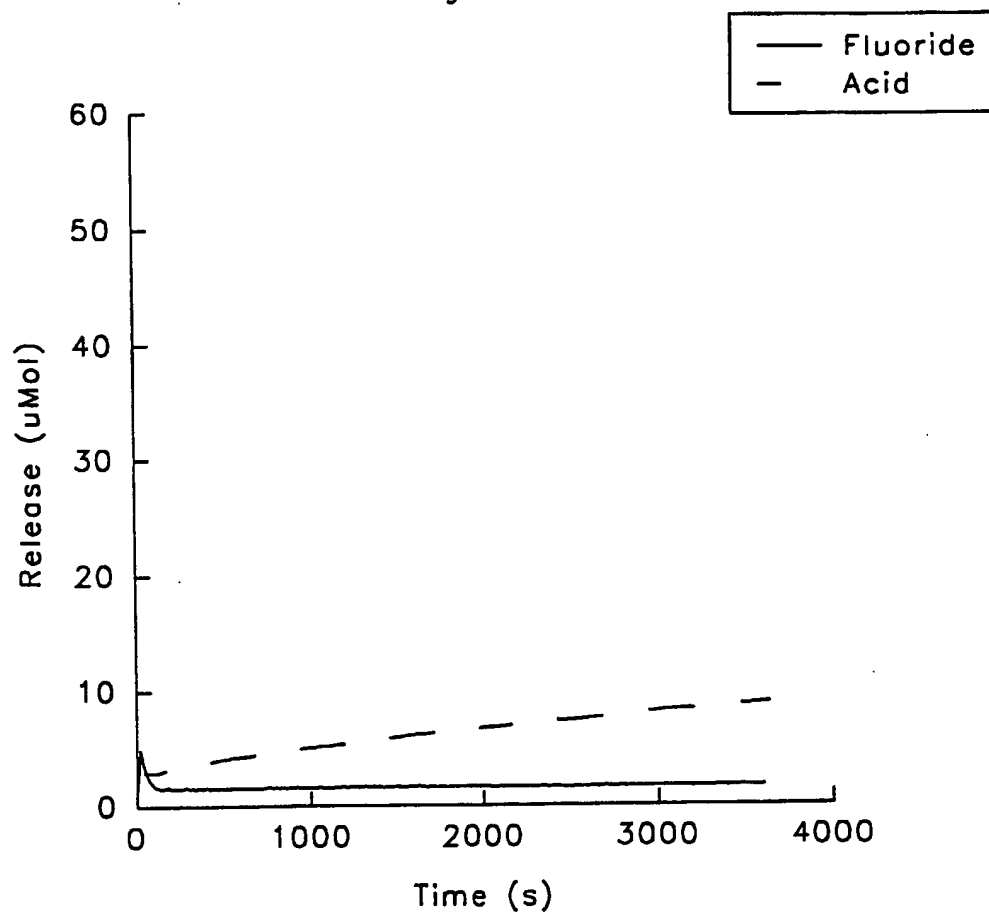
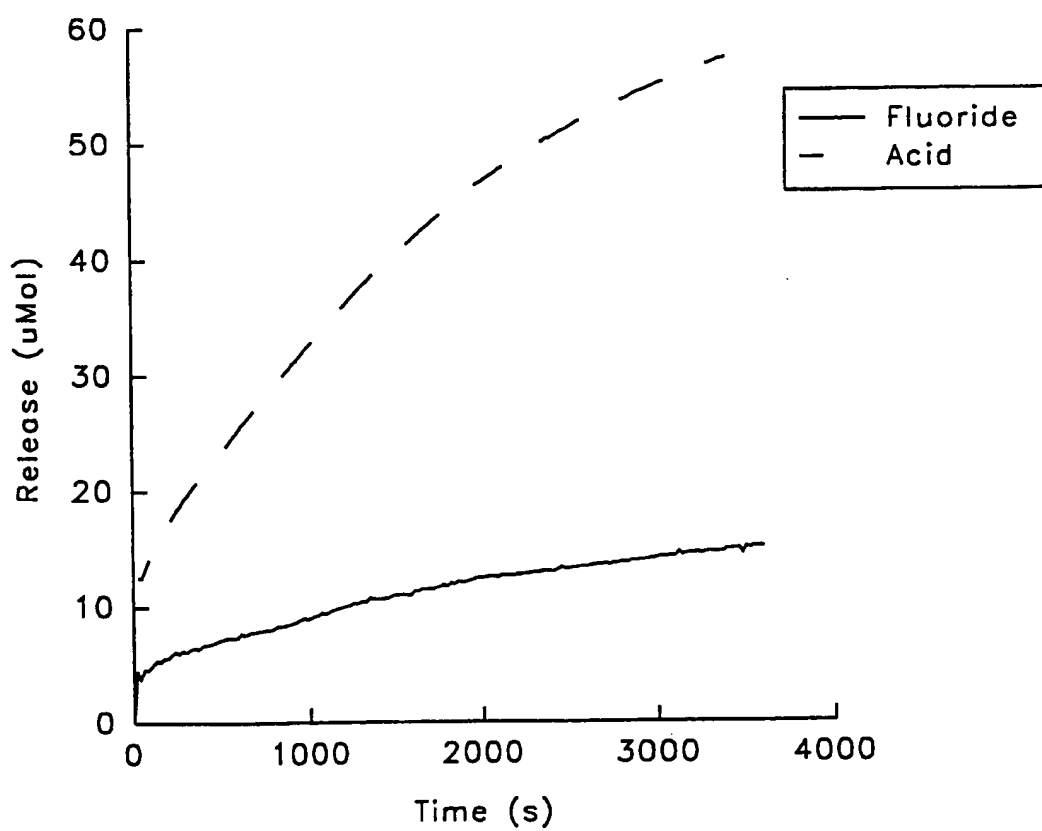


Figure 17



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/00253

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 06058 A (ROYAL FREE HOSP SCHOOL MED ; FRANCIS GILLIAN ELIZABETH (GB); FISHER) 2 March 1995 see abstract; claims 8-11,15-18,22	1-13
X	FRANCIS G E ET AL: "POLYETHYLENE GLYCOL MODIFICATION: RELEVANCE OF IMPROVED METHODOLOGY TO TUMOUR TARGETING" JOURNAL OF DRUG TARGETING, vol. 3, 1996, pages 321-340, XP002058312 see page 323, column 2 - page 324, column 1 see page 325; figures 1-3 see table 1	1-13
A	---	---
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 June 1998

Date of mailing of the international search report

15. 07. 1998

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Fax: (+31-70) 340-3016

Authorized officer

Gonzalez Ramon, N

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/00253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DELGADO C. ET AL: "Polymer derivatized proteins: analytical and preparative problems" PHARMACEUTICAL SCIENCES, vol. 3, 1997, pages 59-66, XP002068522 see page 60, column 2; figures 1,2,4 ---	1-13
X	WO 90 04650 A (ROYAL FREE HOSP SCHOOL MED) 3 May 1990 see page 7-8; figure 1 ---	1,2,4,5, 7-13
X	ZALIPSKY S: "CHEMISTRY OF POLYETHYLENE GLYCOL CONJUGATES WITH BIOLOGICALLY ACTIVE MOLECULES" ADVANCED DRUG DELIVERY REVIEWS, vol. 16, no. 2/03, 1995, pages 157-182, XP002037428 see page 161, column 2, paragraph 3; figure 2; tables 1,3,4 see page 176 - page 177 ---	1,2,4,5, 7-13
X	ZALIPSKY S.: "Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates" BIOCONJUGATE CHEM., vol. 6, no. 2, 1995, pages 150-165, XP002068523 cited in the application see page 151, column 1, paragraph 3; figure 1; table 2 ---	1-3,6-13
X	WO 95 34326 A (KOHNO TADAHIKO ;KACHENSKY DAVE (US); HARRIS MILTON (US)) 21 December 1995 see page 16, line 2-8 see page 19, line 1-7; example 1 ---	1-13
X	EP 0 539 167 A (ORTHO PHARMA CORP) 28 April 1993 see page 5, line 56 - page 6, line 2; examples 2,6 see page 10, line 1-25; claim 21 ---	1,2,4,5, 7-13
A	VERONESE F. M. ET AL: "A comparative study of enzymatic, structural and pharmacokinetic properties of superoxide dismutase isolated from two sources and modified by monomethoxypolyethylene glycol using different methods of coupling" ANNALS N. YORK ACAD. SCI., vol. 613, 1990, NEW YORK, pages 468-474, XP002068524 see page 469, paragraph 3; figure 1; tables 1,2 ---	1-13
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GAERTNER H F ET AL: "SITE-SPECIFIC ATTACHMENT OF FUNCTIONALIZED POLY(ETHYLENE GLYCOL) TO THE AMINO TERMINUS OF PROTEINS" BIOCONJUGATE CHEMISTRY, vol. 7, no. 1, 1996, pages 38-44, XP000646874 see abstract; figure 1 -----	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00253

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 1,2,7-12
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 98/00253

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 1,2,7-12

In view of the large number of compounds, which are defined by the general definition in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application. (see Guidelines, Chapter III, paragraph 2.3).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9506058 A	02-03-1995	EP 0714402 A JP 9504515 T	05-06-1996 06-05-1997
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EP 0539167 A	28-04-1993	AU 668841 B AU 1168495 A AU 658231 B AU 2718392 A CA 2080891 A FI 924747 A JP 5214092 A NO 971085 A NZ 244778 A ZA 9208099 A	16-05-1996 01-06-1995 06-04-1995 22-04-1993 22-04-1993 22-04-1993 24-08-1993 22-04-1993 25-03-1994 20-04-1994

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